Are Tasmanian sweet cherries effective functional foods?

Melanie L Blackhall

Bachelor Human Movement
(Exercise Science)

MPhil (Nutrition Research)

Thesis submitted in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in the School of Medicine,
College of Health and Medicine,
University of Tasmania, Australia

Supervisory Team

Prof Justin Walls

Prof Dom Geraghty

Dr Kiran Ahuja

October 2019

UNIVERSITY of TASMANIA

Declarations

Declaration of Originality

I hereby declare that this thesis entitled “Are Tasmanian sweet cherries effective functional foods?” contains no material which has been accepted for a degree or diploma by the University of Tasmania or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material has previously been published or was written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Melanie L Blackhall Date: 30th October 2019

Authority of Access

This thesis may be made available for loan and limited copying and communication in accordance with the Copyright Act 1968.

Melanie L Blackhall Date: 30th October 2019

Statement Regarding Published Work Contained in Thesis

The publishers of the paper comprising chapter 2 hold the copyright for that content, and access to the material should be sought from respective journals. The remaining non-published content of the thesis may be made available for loan and limited copying and communication in accordance with Copyright Act 1968.

Statement of Co-Authorship

The following people contributed to the publication of work undertaken as part of this thesis:

Candidate -- Melanie L Blackhall, School of Medicine, University of Tasmania

Author 1 -- Prof Justin T Walls, University of Tasmania

Author 2 -- Prof Dom Geraghty, University of Tasmania

Author 3 -- Dr Kiran DK Ahuja, University of Tasmania

Author 4 -- Adjunct Prof Noel W Davies, University of Tasmania

Author 5 -- Ms Rachael Berry, University of Tasmania

Contribution of work by co-authors for each paper:

Paper 1: Located in chapter 2

Blackhall ML, Berry R, Davies NW, Walls JT. Optimized extraction of anthocyanins from Reid Fruits' Prunus avium 'Lapins' cherries. Food Chem. 2018;256:280-5.

Author contributions:

- Candidate was the primary author and contributed 80% to the conceptualization, planning and execution of the research design.
- Author 1 contributed to the conceptualization, planning and execution of the research design
- Author 4 contributed through analysing samples and providing input to research methodology.
- Author 5 contributed significantly to execution of laboratory trials and contributed to drafts of the manuscript.
- All authors contributed to drafts and final proof reading of the manuscripts.

Paper 2: Located in chapter 3

Anthocyanin content of sweet cherries: differences in fruit grade, cultivar, seasonal variation and storage conditions

- Candidate was primary author and contributed 80% of the research design and planning, execution and data interpretation, and subsequent publication.
- Authors 1 and 5 contributed to significantly to research planning and design, author 5 contributed significantly to research execution and data collection.
- Authors 1, 2 and 3 contributed significantly to data analysis and interpretation.
- All authors contributed to drafts and final proof reading of the manuscripts.

Paper 3: Located in Chapter 4

Sweet cherry anthocyanins reduce weight gain and inflammation in high fat fed mice.

- Candidate was the primary author and contributed 80% of the planning and

execution of the research project and subsequent publication.

- Author 1, 2 and 5 contributed significantly to research design and data analysis.
- Author 5 contributed significantly to laboratory trials, execution and data analysis.
- Author 3 contributed to data analysis and interpretation.
- All authors contributed to drafts and final proof reading of the manuscript.

We, the undersigned, endorse the above stated contribution of work undertaken for each of the published (or submitted) peer-reviewed manuscripts contributing to this thesis:

Signed:

Melanie Blackhall	Justin Walls	Lisa Foa
Candidate	Primary Supervisor	Head of School (Acting)
School of Medicine	University College	School of Medicine
University of Tasmania	University of Tasmania	University of Tasmania
Date: 5th November 2019	**5th November 2019**	**5th November 2019**

Statement of Contribution to Thesis

The thesis comprises three large research investigations that are broken into a number of sub-components and the candidate completed the majority of the experimental work and is first author on the manuscripts. Specialised assistance was provided as detailed below:

- Justin Walls (University College, University of Tasmania): Advised on study design, assisted with data analysis and manuscript revisions.
- Dom Geraghty (College of Health and Medicine, University of Tasmania): Advised on study design, animal ethics, data interpretation and manuscript revisions.
- Kiran Ahuja (College of Health and Medicine, University of Tasmania): Contributed to statistical analysis, data interpretation and manuscript revisions.
- Rachael Berry (College of Health and Medicine, University of Tasmania): Contributed to design, data collection and analysis, execution of trials and manuscript revisions.

Additional technical support was provided by:

- Noel Davies (Adjunct Professor, University of Tasmania): Contributed to sample analysis and interpretation.
- David Nichols (Central Science Laboratory, University of Tasmania): Contributed to sample analysis and interpretation.
- John Cardinal (Cardinal Bioresearch): Contributed to sample analysis.
- Nick Owens (Reid Fruits): Contributed technical aspects of cherry production.

Photos included in the thesis

Photos that do not indicate a source were taken by the candidate and included in the thesis to provide context to the document.

Statement of Ethical Conduct

The research associated with this thesis abides by International and Australian codes of human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University (Tasmania) Network; approval numbers A0013383 and A0015582.

Melanie L Blackhall Date: 30th October 2019

List of Publications

Blackhall, ML, Berry, R, Davies, NW, Walls, JT. Optimized extraction of anthocyanins from Reid Fruits' Prunus avium *Lapins* cherries. Food Chem. 2018; 256:280-85. Status: Published

Blackhall, ML, Berry, R, Ahuja, KDK, Geraghty, DG, Walls, JT. Sweet cherry anthocyanins reduce weight gain and inflammation in high fat fed mice. Dec 2018. Status: Currently under review

Blackhall, ML, Berry, R, Ahuja, KDK, Geraghty, DG, Walls, JT. Anthocyanin content of sweet cherries: differences in fruit grade, cultivar, seasonal variation and storage conditions. Status: In preparation for submission

List of Conference Presentations

December 2018: **The Nutrition Society of Australia ASM** (Canberra Australia), In vitro investigation of sweet cherry extract (appendix 8).

November 2017: **Asia Pacific Conference on Clinical Nutrition** (Adelaide, Australia), Anthocyanins derived from sweet cherries reduce obesity markers in high fat fed mice.

November 2016: **The Nutrition Society of Australia ASM**, (Melbourne, Australia), The impact of storage conditions and growing season on the anthocyanin content of sweet cherry varieties.

December 2015: **Joint Scientific Meeting of the Nutrition Society of New Zealand and Nutrition Society of Australia** (Wellington, New Zealand), 'Physiological'

temperature found to be prime for extracting sweet cherry anthocyanins (appendix 9).

December 2015: **Joint Scientific Meeting of the Nutrition Society(s) of New Zealand and Australia** (Wellington, New Zealand), Ripe for the picking: Anthocyanin content of waste fruit versus first grade fruit in Australian sweet cherries.

May 2013: **Fruit Growers Tasmania Annual Conference** (Hobart, Tasmania) Clinical Trials in Tasmania: Health Benefits of Anthocyanins.

Funding Support and Grants

Funding for the life of the project was gratefully received from Reid Fruits Pty Ltd. Additional funding was sought and granted through a Department of Business, Innovation Connection grant – in partnership with industry group Essential Oils of Tasmania.

I was also successful in receiving grants from School of Medicine, College of Health and Medicine, University of Tasmania – conference travel scheme. Grants were received in the following years:

- School of Medicine, University of Tasmania, Conference Travel Scheme, to attend Joint Scientific Meeting of the Nutrition Society of New Zealand 2015.
- School of Medicine, University of Tasmania, Conference Travel Scheme, to attend The Nutrition Society of Australia ASM, 2016.
- School of Medicine, University of Tasmania, Conference Travel Scheme, to attend Asia Pacific Conference on Clinical Nutrition, 2017.
- School of Medicine, University of Tasmania, Conference Travel Scheme, to attend The Nutrition Society of Australia ASM, 2018.

Acknowledgements

After writing this thesis and being accustomed to putting a volume of words to a page one would think the acknowledgements would be an easy final step. Yet the words that I attempt to craft don't seem to do it justice. My first thanks go to my supervisors, Justin, Dom and Kiran. It's been quite the "journey" and even though I detest that word it is best for this purpose. With multiple health glitches and big life events you have all been there. Kiran, when you joined the team you brought a critical eye which was welcomed and is appreciated. Your attention to detail, particularly in the stats and writing (consistency!) meant the final product is finessed and ready. Dom, you have been a keen supporter from the start. Always finding ways to lift me up when I needed it (and to pull things in when I got a bit verbose) your generosity over last year of writing has had a big IMPACT (see what I did there). And finally Justin, through all the contract negotiations, board meetings, presentations, big ideas, failed assays and successful trials you were there with wisdom, friendship and sometimes bad jokes. Your support of me and belief in the research story I was trying to craft meant I didn't give up, even at times when things went wrong, and I will be forever grateful.

To Rach…we made it!! There is no question that I would not have made it to the end without you. There were so many challenges along the way, but our mantra got us there, and guess what we were right…everything will be fine. Now let's celebrate!

I will be eternally grateful to my research partners for believing in my ideas and having the confidence to back me. Without your support the research may not have happened. To Tim, Lucy and Nick Owens at Reid Fruits, thank you.

The last bunch are the trickiest, friends and family. SO many friends have helped

along the way and this PhD is for all of us. For the support, picking up and looking after the girls, bringing food, laughing and listening to my ramblings (over coffee or gin) and being there for me, thank you. I can't name you individually (or I'd need another 100 pages) but you know who you are.

To my family. JJ and Fleaby you have ridden every step of this with me and I know I could not have made it without your support. I am proud to have completed this for you as much as me. To Lellsa and Lukey et al, for listening, offering support and at times offering to be a strong advocate, thank you.

To my daughters India and Zali, you are the best young people I know, and for that I am proud. For the missed performances, my absence and for you having to witness the pressure of it all, this makes it all worth it. You are my inspiration and knowing that you will be strong and independent women makes me happy. You are going to live in a world where you can do anything you want, so find something you love, work hard and follow your heart. Toddy, the husb of all husbs, there aren't enough words. You have put things on hold for me and you have held our family together through all of it. Your patience, confidence in me, resilience when I was was overwhelmed and stressed, for ensuring the girls were ok and holding my hand or pouring us a gin, you are everything. No more study, I promise.

Whilst the ideas for this research were cooked up in the chemo chair, there have been many supporters that have encouraged me to bring this to completion, and for that sage advice I am grateful. It appears I CAN WRITE! Insomnia, it's time for you and I to break up. It's been a ride, I am done, and I am thankful.

Table of Contents

List of Figures

List of Tables

List of Appendices

List of Common Abbreviations

TAC – Total anthocyanin content

SCA – Sweet cherry anthocyanin

UPLC – Ultra-performance liquid chromatography

WAT – White adipose tissue

HCL – Hydrochloric acid

LPS – Lipopolysaccharide

General Abstract

Are Tasmanian sweet cherries effective functional foods?

Background and Aims

Sweet cherries or Prunus avium L. are a rich source of anthocyanins, which are bioactive secondary metabolites of flavonoids. Determining the bioactivity or functional capacity of sweet cherries and how we can efficiently harness waste fruit to treat disease using food waste is the crux of this thesis. There are two significant challenges facing the human race and both are driven by excess - obesity and food waste. The prevalence of obesity-related inflammation and resulting morbidity and mortality has dramatically increased in recent times. Globally, this equates to 39% of the world's adult population in an unhealthy weight range. Pharmaceutical treatment options have been limited and ineffectual, with many of the drugs removed from the market due to significant side effects. Both the scale of the obesity epidemic and the severity of the side effects from drug treatments, necessitate alternative options for treatment.

Bioactive components in food have been identified as potential modulators of health, where their functional capacity is harnessed to attenuate disease processes. Anthocyanins have been identified as beneficial due to their antioxidant properties, yet it is potential anti-inflammatory anti-obesity actions that warrant exploration. High anthocyanin fruits such as sweet cherries, may potentially attenuate disease whilst reducing the parallel burden of food waste. The global burden of food waste is also of

concern with significant investment occurring to develop methods of harnessing the waste to reduce reliance on landfill. Annually, global cherry production is estimated at 3.7 million tonnes, but 20 – 50% of that fruit is usually deemed not fit for sale. To determine whether cherry waste fruit could be used for other purposes, comprehensive quantification and characterisation of the fruit was needed to be undertaken. The studies that make up this thesis were first aimed at optimising the extraction of anthocyanins from waste fruit at the highest possible yield. Further stability and reproducibility experiments were undertaken to determine the effect of storage on total anthocyanin content (TAC) and variation within fruit. This allowed examination of the functional activity using *in vitro* (cell culture) and *in vivo* (animal feeding trials) experiments.

Methods and Results

The effect of process parameters on the extraction of anthocyanins from Prunus avium L. 'Lapin' was determined. Time, temperature, solvent type, solvent to solute ratio and fruit size for assay were all tested. The optimal conditions for extraction were fruit homogenised in acidified ethanol, macerated for 90 minutes, at 37^{o}C, at a ratio of 10ml/g (solvent:solid) resulting in the greatest yield of anthocyanins and a three-fold increase in amount extracted prior to optimisation steps.

Subsequently, a comprehensive examination of anthocyanin content across different growing seasons, cultivars and grades of fruit, plus the effect of post-harvest storage conditions was conducted. The Prunus avium L. 'Kordia' cultivar was shown to have a significantly greater anthocyanin content compared to all other cultivars (consistent across seasons), with a value of 873mg/100g fresh weight compared with an average of 244mg/100g fresh weight for other cultivars. Post-harvest storage conditions at 3,

6, 12 and 24 months showed that storage at -20°C resulted in significantly less anthocyanin remaining than at -80°C. Indeed, with as little as 4.4% of TAC remaining after 24 months at -20°C, if preservation of anthocyanin content is a priority, storage conditions must be considered. Following a retrospective analysis of weather patterns in conjunction with the fruit specimens assayed across seasons, 'ideal growing conditions' that result in less waste fruit were tentatively proposed.

The final series of experiments investigated the anti-inflammatory properties of the sweet cherry (Lapin cultivar) extract. RAW264.7 cells were stimulated with lipo-polysaccharide (LPS) and when pre-treated with sweet cherry anthocyanin, there was significant reduction in inflammatory markers IL10 and GM-CSF. To measure anti-inflammatory effect *in vivo*, a murine supplementation trial was undertaken. In the trial, high fat fed C57BL/6 mice were supplemented with sweet cherry anthocyanin (SCA) to determine whether obesity and inflammation could be prevented or reversed. After 6 weeks of supplementation in the prevention trial, there was 19% less weight gained in the treated mice versus matched controls (this was not observed in the reversal trial). There were also significant outcomes in relation to inflammation in both the prevention and reversal trials, with SCA treated mice exhibiting reduced inflammation than matched controls. If these results were translated to clinical terms, a weight reduction of this level and reduced inflammation would be considered clinically significant outcomes.

Premium grade Sweetheart cherry

(> 34mm size)

Waste (small) Lapin cherries

(<28mm size)

Figure 0.1 Cherry size is one example of criteria that dictates cherry to be deemed premium or waste fruit.

Conclusions

Through optimisation from sweet cherries, a highly efficient, high yield method for extracting anthocyanins was developed. This had the added and unexpected finding of an optimal extraction temperature (37^{o}C) which is particularly important for biological experiments. That waste fruit was found to have a higher total anthocyanin content than premium grade fruit holds great significance for industry. It provides justification and economic rationalisation for investing in strategies to re-purpose the waste. As a sole outcome for industry and consumers, this finding is heartening.

As sweet cherry anthocyanin significantly reduced the rate of weight gain and the associated chronic low-grade inflammation induced by a high fat diet, this fruit provide a potential non-drug, adjunct therapy for obesity and inflammation. As waste fruit was found to have higher anthocyanin than premium fruit, and there was significant effect in both cell and animal studies, it does suggest waste can be used to treat disease.

Chapter 1: Thesis Introduction and Overview

Foods added to the diet that improve overall health and wellness, in addition to providing nutrient benefit, are called functional foods (1, 2). Historically, nutrient profile has been the primary reason for the consumption of foods, with more emphasis in recent times on foods with high bioactivity (3-5). Flavonoids are secondary metabolites that exhibit high bioactivity and are categorised further into sub-groups including anthocyanidins (or their glycosylated form called anthocyanins), anthoxanthins, flavanones, flavanonols, flavans and isoflavnoids (6, 7). The bioactivity of a compound can be measured through a range of assays that measure the antioxidant, antimicrobial, anti-inflammatory and other actions of the compound (8, 9).

To be able to determine if sweet cherries meet the definition of a functional food, investigation into the bioactive profile of the fruit is required (10). Cherries are known to be a rich source of anthocyanins and the premium grade cherries contribute $164 million to the Australian economy annually (11). A significant proportion of the locally grown fruit is destined for overseas export markets (11). Total cherry fruit exports for Australia in 2016 was 5,593 tonnes with a value of $76 million, with Tasmania producing more than half of this 2,872 tonnes with a value of $50 million (11). Whilst small on a global scale, there is real value for the domestic economy. Unfortunately, up to 50% of the annual crop is deemed waste, with minimal repurposing. However, waste from other fruits is being recycled into value added products and used for a variety of applications including silage for animal feed, as natural food colourants and in the food industry to increase fibre content of foods (12-15). The aims of the studies described in this thesis were to:

(1) determine if waste fruit provides a concentrated source of anthocyanins,

(2) investigate the factors that influence the yield of anthocyanins in extraction and

(3) determine whether anthocyanins possess anti-inflammatory properties in vitro and in vivo.

This thesis will therefore explore the following concepts in both premium grade and waste sweet cherries: Firstly, a standard approach to the identification, quantification and optimisation of extraction will be developed. This will enable efficient extraction of anthocyanins to ensure maximum recovery/ yield. Secondly, an in-depth examination of the fruit and factors that may influence bioactive components and the yield of anthocyanin will be determined. Lastly, cherry extract functionality will be tested through a series of in vitro and in vivo experiments to provide understanding and context for application of sweet cherries as a functional food. These seminal findings, with broad appeal, will be tied together to provide a practical understanding of the bioactivity and functionality of Tasmanian sweet cherries.

1.1 Background

1.1.1 Functional food

A functional food is a food that provides a physiological benefit or reduces the risk of disease, in addition to its primary nutritional function (16). With the advent of modern medicine, the creation of pharmaceuticals and pharmacotherapy has meant foods as therapeutic agents have been overlooked until recently (3). Emerging evidence has highlighted the potential for foods to add benefit to health without the side-effects experienced with drug treatments (4, 17, 18). At present, functional foods have a demonstrated role in mitigating the effects of diseases such as obesity (19), metabolic

syndrome (20), immune dysfunction and inflammation (21). They have also been reported as central to improving and maintaining geriatric nutrition (22), improving intestinal health and supporting gut microbiota (23, 24).

Functional foods exist in many forms including wholefoods, fortified or enriched foods and modified foods (25). The functional capacity and bioactivity of these foods arises from biologically active compounds (phytochemicals) contained within (26, 27). The activity of many phytochemicals from fruit has been determined through both in vitro and in vivo studies (28-35). However, in-depth investigations of the bioactive role of sweet cherry anthocyanins from waste and premium fruit has not been undertaken to date.

Whilst the concept of functional food was likely born out of a desire for better health, the consumer is now driving the functional food/ nutraceutical market (25, 36, 37). It is clear that as consumers have become more interested in the provenance of food and they are now driving a contemporary pattern of consumption (38, 39). Consumption of food from the local area is considered valuable and has led to an increased demand for locally sourced and value-added products. However, consumerism in this sense is dichotomous, as the consumer desires perfectly formed fruit (40). Whilst consumers desire functional food and with connection to provenance, their requirement of first grade fruit free from blemishes, splits or cracks is likely influencing the portion of fruit rejected by the market. As such it is this evolution that has driven significant commercial investment in developing and understanding bioactive foods compounds and their potential to attenuate disease (41). Emerging evidence has highlighted the health benefits derived from consuming fruit with high levels of anthocyanins (flavonoids) (28, 32, 42-53). These benefits include control of blood glucose levels, reduced cancer growth and improvement in cardiovascular

disease profile through improved blood vessel wall vasodilatation.

1.1.2 Anthocyanin bioactivity and extraction

Anthocyanins are water-soluble pigments that form part of a subclass of polyphenolics and are responsible for the dark purple, blue and red colour of many plants, flowers, fruit and vegetables (6, 54-57). They are a sub-group of flavonoids, which is one of classes of the secondary metabolites, and are present in varying amounts across fruit species (6, 58-60).

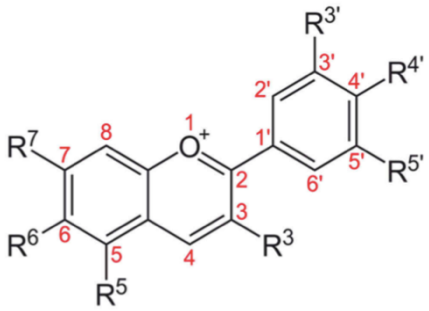


Figure 1.1 Basic anthocyanin structure.

As taken from "Anthocyanidins and anthocyanins: coloured pigments as food, pharmaceutical ingredients, and the potential health benefits", Khoo et al 2017 (8).

As there are over 600 anthocyanins identified in nature, it is expedient that the anthocyanins be categorised further. Anthocyanins are glycosylated form of anthocyanidins and the six principal aglycon (anthocyanidins) forms most abundantly found in plants are pelargonidin, cyanidin, delphinidin, peonidin, petunidin and malvidin (60-62).

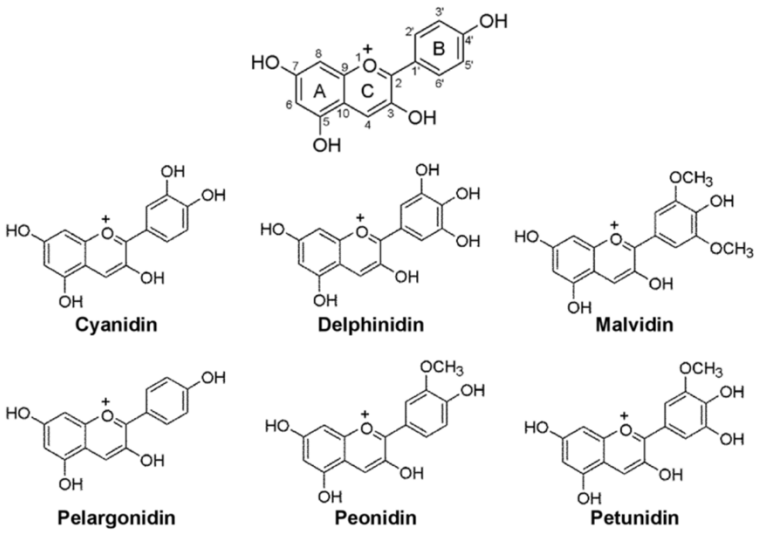


Figure 1.2 Major anthocyanidins in plants.

As taken from "Anthocyanidins and anthocyanins: coloured pigments as food, pharmaceutical ingredients, and the potential health benefits", Yuan et al 2012 (63).

Of interest is the investigation of anthocyanins in fruit species. These metabolites have been studied across many fruits however, the majority of anthocyanin research has been undertaken in blueberries, raspberries and tart cherry (Prunus cerasus) cultivars (34, 51, 53, 64-71). Whilst the bioactivity and profiling of sweet cherry (Prunus avium L.) cultivars is the subject of current work by other investigators (27, 40, 50, 52, 72-99), a comprehensive understanding of the bioactivity and function of the fruit is lacking.

The physiology of fruit is a pertinent consideration, as the growth and development of the fruit will affect its bioactivity at maturity (100). Phytochemistry (plant chemistry) is the study of chemicals synthesised in plants for example, the development of pigmentation in the fruit, which correlates to the flavonoid content of the fruit (101). The level of pigmentation or depth of colour provides protection against microbial insults, provides natural UV protection to plants and attracts pollinators to ensure

species survival (101-103). For most fruit and plant species, the high anthocyanin/colour is concentrated in the skin (89, 104). However, cherries are unusual in that they are coloured from the exocarp through mesocarp to endocarp ("skin to seed") suggesting a much higher overall secondary metabolite content.

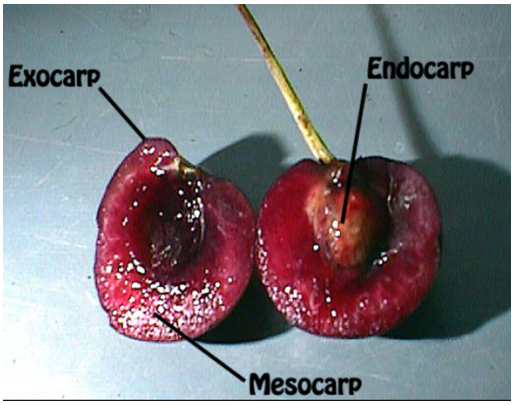


Figure 1.3 Dissection of cherry showing layers of the fruit – the exocarp, mesocarp and endocarp.

As taken from Miami University index of images, showing the anatomy of different fruit species (105).

The bioactivity of anthocyanins is controlled by their molecular sub-group (106). Of the secondary metabolites, cyanidin and its derivatives are the most common and are thought to contribute approximately 90% of the total anthocyanin profile in sweet cherries (6, 58). At low pH (<2), the compound appears red, at a pH of between 2 to 4 the compound is blue and in alkaline conditions (above pH 7) the anthocyanins are degraded (58). The pH is therefore important to consider when understanding and applying techniques of extraction and purification to ensure the profile that is extracted has the bioactivity remaining required for function (107). Extraction techniques historically have recovered a low yield of anthocyanin, which is indicative of the unstable nature of the compound (58). Pressurised liquid extraction (PLE) (108-110), microwave assisted extraction (MAE) (111-113) and ultrasound assisted extraction

(UAE) (111, 114, 115) are techniques widely used in the extraction of anthocyanins in addition to the traditional maceration method . Solvents commonly used are methanol or acetone acidified with hydrochloric, acetic or formic acid, resulting a difference in yield of extraction/ efficiency (116). Solvent-based extraction involves macerating or homogenizing a solute in solvent with the resultant bioactive component in the solvent to be evaporated and analysed (116). Whereas microwave- and ultrasound-assisted extraction reportedly increase efficiency of the extraction yield. Whilst they require less solvent to extract through the use of microwave and ultrasound assisted technology to heat and process the samples in solid-liquid phase, this is a more specialised technique (111). However, given the applied nature of this research, investigation of parameters needs to reflect the technology that would be accessible to industry to bring any findings to commercial realisation.

Extraction and subsequent screening for the bioactivity in anthocyanin rich fruits through ORAC, ABTS and FRAP methods has enabled limited understanding of antioxidant function (50, 67, 72, 117). However, when considering the bioactivity of fruit as measured by total anthocyanin content, there are a number of factors that remain to be determined. The phytochemicals responsible for creating physiological change, the effect of these compounds on cytokine expression as a surrogate of inflammation (*in vitro* and *in vivo*) and susceptibility of these compounds to degradation post-harvest all warrant investigation.

Sweet and sour cherries, blueberries, blackberries, strawberries & red raspberries have been identified as being a rich source of anthocyanins (73, 74, 91). Reported anthocyanin levels range from 14.7 mg/100g fresh weight in red raspberries, through to 58.6 mg/100g fresh weight in blackberries (91). However, as will be shown in Chapter 2, the concentration of anthocyanin in sweet cherries ranges from

6.21mg/100g to 244mg/100g fresh weight for different varieties whereas for sour cherries, the maximum previously recorded is 98.4 mg/100g fresh weight (72, 74, 82).

1.1.3 Cherry anthocyanins: crop, cultivars, waste & seasonal effect

Primary production of anthocyanin rich fruits is burgeoning in Australia, particularly cherries, blueberries, raspberries, strawberries and red skinned apples (11). Tasmanian sweet cherries are a valuable commodity and attract a high price in the international export market (118). The volume of production and the inherent value of this crop to the local economy, provide the perfect milieu to quantify and explore functionality of anthocyanin rich cherries.

Figure 1.4 Large size cherries shown with reference to a 50-cent coin.

Photo: courtesy of Reid Fruits

The *Prunus avium* '*Lapins*' cherry cultivar is a crossbreed of the *Stella* and *Van* varietals, that are self-fertile and accounts for approximately 50% of the total commercial cherry yield in Tasmania (119). In Australia, there are approximately 80 different varieties grown, with ten of the varieties grown in Tasmania discussed and compared throughout this thesis (appendix 1). The temperate climate of the southern region of Australia provides optimum growing conditions for *Prunus avium* cultivars,

with seasonal factors paramount to growth (81). Cherry growth (figure 1.5) can be fickle and is determined by the perfect matrix of temperature, rainfall and sunlight.

During the winter dormancy period, the fruit requires low temperatures (chilling/ cold-set) to ensure adequate bud formation (119). The onset of Spring heralds tree bud swell, whereby the bud that was set during winter begins to grow prior to blossoming. During the spring months, little rainfall and the absence of severe frosts are required to ensure maximum pollination and fruit set are achieved (119). If there are severe frosts and low ground temperature during the period of cherry blossom, there is the potential for frost injury and reduced fruit formation, Late spring and early summer is the fruit growth and maturation period, where fruit develops from the bud and blossom that has developed in months prior. Crucial periods of impact from rainfall are in the spring blossoming and early summer growing and harvest season where fruit is developing. Rainfall during this period has the potential to prevent fruit growth and cause subsequent damage to fruit at maturity.

Figure 1.5 Growing cycle of cherries

Photo: courtesy of Reid Fruits

Whilst the production of sweet cherries in Australia is strong, there is significant volume that does not make it to market, and ends up as waste (11). Globally, reducing waste is of critical concern, with significant investment being made to develop strategies to re-purpose and harness waste products (120, 121). Waste from many different fruit

species is being utilised to develop new products that will reduce the amount of fruit discarded. Commonly, fruit that has splits, cracks, blemishes or increased softness are defining characteristics that make the fruit less desirable and relegate it to the seconds/ waste fruit bins (88, 122, 123). Cherry waste is currently of little economic value to growers and insight gained from this study may add worth to the currently under-utilized crop.

The cherry harvest and consumption season in Australia is relatively short, approximately one hundred days in length, which commonly requires fruit to be stored prior to processing. Research into the impact of postharvest processing and storage conditions on bioactive compounds in blueberries, blackberries and raspberries has been undertaken. Researchers found degradation of anthocyanins was greater at higher temperatures and reported losses in the range of 28% to 59% (65, 124-126).

Storage at temperatures between the 0 to 30^{o}C has been shown to reduce cherry lifespan and quality through an increase in metabolism and respiration (123). Storage temperature has also been found to have a significant effect on the rate of water loss in sweet cherries (98). Water loss is one of the main limiting factors in post-harvest lifespan, and has prompted many producers to circumvent this risk by reducing the pulp temperature of cherries to between -4^{o}C and -1^{o}C following harvest (98). Whilst there appears consensus on prolonging fruit life through avoiding high temperatures, it remains to be determined how storage at this temperature affects anthocyanin content. Research to date is suggestive of anthocyanin content being influenced by both pre and post-harvest factors. As such, the effect of weather and environmental stressors, storage conditions and extraction methods on anthocyanin (77, 78, 89, 111, 119) will be explored throughout this body of work.

Figure 1.6 Mounds of waste cherry fruit in Tasmania.

1.1.4 Obesity, inflammation & anthocyanin

Obesity (in particular due to white adipose tissue, WAT) is associated with a state of chronic, low-grade inflammation not related to infection (127). Adipocytes from WAT are large in size, with limited insulin receptors for promotion of glucose uptake, but have high levels of beta-receptors that promote inflammatory marker secretion (127). As they expand in size to continue to uptake glucose, lipolysis is promoted, resulting in macrophage infiltration of the WAT (128). During lipolysis, macrophages secrete cytokines TNF-α and IL-6, which promote macrophage activation and cause chronic low-level inflammation (129). Over time, the macrophages differentiate and fuse multinucleate cells that persist for extended periods of time producing more TNF-α and IL-6 (130). WAT appears to be both a source of inflammation and a target for macrophages. The World Health Organisation (WHO) estimates that more than 1.9 billion adults are overweight or obese. With that number continuing to rise (131), the burden of living in a state of metabolic inflammation increases in magnitude. Obesity-related inflammatory disease is the catalyst for developing type 2 diabetes and dramatically increases the risk of cardiovascular disease (CVD), of which the underlying mediator is an increase in inflammatory and pro-inflammatory mediators, including IL-6, IL-8, IL-1β, CRP & TNF-α (132).

To address the extent of the obesity crisis globally, there are a raft of anti-obesity medications currently prescribed. The drugs effectively reduce weight by altering appetite, metabolism of macronutrients or reducing consumption of calories (133). However, these medications have been linked to significant side effects and complications, including gastrointestinal and mood disorders (17, 18, 134) causing many to be removed from the market (135, 136). It is the severity of these side-effects that has prompted research into non-drug treatments for obesity.

Obesity, cardiovascular disease, metabolic syndrome and respiratory diseases all stem from an increased inflammatory state and the measurement of this inflammation and is now recognised as one of the major predictors in the development of disease (137, 138). Inflammation and the consequences of inflammatory disease are a major health issue for Australia and other western countries. Inflammation is the non-specific immune response that occurs as a result of an insult/ injury and is classified as either acute or chronic. Infiltration of innate immune system cells, including macrophages and neutrophils, characterise the acute phase response. Macrophages also contribute to chronic inflammation by differentiation and promotion for the infiltration of T lymphocytes (138). In cases of prolonged or chronic low-grade inflammation, the inflammatory cascade of events leads to an increased prevalence of many diseases.

For more than two decades, epidemiological studies have identified an association between the regular consumption of fruits and vegetables and improved health outcomes, particularly in relation to diseases caused by oxidative stress (139-143). More recently, anthocyanins have been identified as the chemical compounds central to a range of improved health outcomes (144, 145). Whilst traditionally anthocyanins have been used as natural colourants in the food industry (146), their use as functional foods to treat disease grows (47, 64). Specifically, anthocyanins derived from blueberries had a positive effect on obesity measures in a murine model as shown through reduction in epidydimal fat pad weight (53), and a blackberry-blueberry mix attenuated obesity-derived inflammation in vitro (44). Whilst anthocyanins are known powerful antioxidants (62, 70), preliminary work into their capacity to improve indices of metabolic syndrome and gout is encouraging. Specifically, body mass, blood pressure and glucose tolerance were improved following chronic anthocyanin dose in high fat fed mice (29) and a reduction in the incidence of gout flares was evident after

consumption of cherry juice (147). However, the effect of anthocyanins on obesity-derived inflammatory disease and the underlying mechanisms by which inflammation is attenuated are yet to be fully elucidated.

The majority of research that has been undertaken in anthocyanins is from berry fruits or tart cherry varieties (*Prunus cerasus*) with limited research undertaken into the health impact of sweet cherry (*Prunus avium L*) cultivars (94, 148, 149). The potential to understand the functional effects of the sweet cherry anthocyanins on obesity-derived inflammation as explored through in vitro then in vivo studies forms the basis of this body of work.

1.2 Research aims

The overall aim of this body of work is to quantify, characterize, and investigate bioactivity of sweet cherries, by extracting the anthocyanins and assessing some of their functional properties through *in vitro* and *in vivo* studies.

The specific objectives of this research that are presented in this thesis are to:

- Develop a method for extracting, identifying and quantifying sweet cherry anthocyanins.
- Ascertain whether or how anthocyanin content varies across cultivars, seasons and grades of fruit and whether it is impacted by post-harvest storage conditions.
- Determine the functional effects of sweet cherry anthocyanins using *in vitro* and *in vivo* studies of obesity-derived inflammation.

1.3 Significance of the research

The significance of this body of work is multifaceted. It explores optimization of extraction parameters to enable high recovery yield of anthocyanins. This allows accurate quantification of the compounds and application of the biologically active anthocyanins and to determine impact they have on disease. In addition, insight provided allows clearer understanding of the waste cherry as a commodity which contributes to the economic strength and viability of the Tasmanian and Australian fresh fruit market.

Chronic inflammation is the basis of many of the most debilitating diseases for the Australian population and that of the Western world. The possibility that anthocyanins

could attenuate inflammation has the potential to inform both treatment and prophylactic therapies for obesity-related chronic inflammation. Whilst research into sweet cherries effect in oxidative stress and fatty liver disease has been undertaken, understanding how fruit might attenuate disease is warranted as there is paucity of research in this area. This project will help determine whether anthocyanins could be prescribed as a supplement to obese individuals or those at risk of developing obesity. One outcome of this study has the potential to provide a novel alternative therapy in the treatment of chronic inflammatory disease with widespread impact.

However, before studies of functionality can be undertaken and in order to maximise potential yield of anthocyanin(s), an investigation of the factors that affect anthocyanin levels at fruit maturity is needed. This quantification to better understand the profile of cherries grown in Australia may have the potential to influence primary production. Specifically, it is of interest to understand differences across cultivars, variation between seasons and post-harvest storage conditions. Investigation of weather patterns and seasonal influences may help to determine indicators during the winter fruit set and spring blossoming periods that would enable growers to better predict the outcomes for the summer picking season. This in turn would enable fruit to be utilised with efficiency and for producers to prepare for dealing with large volumes of waste.

The gaps in the literature that are of interest to this body of work include the link between high density bioactive compounds in waste fruit, the ability when applied to prevent and reverse disease (inflammation and obesity) and the transfer of this benefit to create a financially viable resource for primary producers.

1.4 Thesis organisation

This doctoral thesis contains a description of a series of experiments and exploration aimed at characterising, quantifying and measuring the functional properties of anthocyanins from Sweet Cherries (*Prunus avium L*). This understanding will provide a depth of knowledge from the farmgate through to the laboratory and commercial outputs. The influence and potential application extend from the primary producer through to the end consumer, with identified health and commercial benefit.

Chapter 1: Is a general introduction and background themes with rationale as to the aims and structure of the thesis and body of work.

Chapter 2: The first series of experiments determined the conditions and techniques involved in characterising and extracting bioactive compounds from sweet cherries. This involved a serial approach to identify the most effective and optimised manner to extract the highest yield from the fruit. Factors optimised included solvent type and concentration, solvent: solute ratio, temperature, time and fruit processing size and conditions (research objective 1). An edited version of this manuscript was published as:

Blackhall ML, Berry R, Davies NW, Walls JT. 2018. Optimized extraction of anthocyanins from Reid Fruits' *Prunus avium Lapins* cherries. Food Chemistry, pp.280–285.

Chapter 3: Involves a series of experiments aimed at characterizing sweet cherries, through experiments of pre and post-harvest conditions. The fruit was investigated to determine difference across cultivar, grade of fruit, seasonal variation (which included a retrospective analysis of weather patterns and conditions) and post-harvest storage

conditions (research objective 2).

An edited version of this manuscript has been prepared for publication to be submitted to a peer reviewed journal.

Chapter 4: The fourth chapter contains series of studies exploring the functional properties of the anthocyanins. In vitro and in vivo experiments explore the effectiveness of the extract in physiological change (research objective 3).

An edited version of this manuscript is under review in a peer reviewed journal.

Chapter 5: The final chapter provides the realised context for application and use of anthocyanins derived from sweet cherries. In a time where economic rationalisation of farming and an individual's concern about maintaining good health are parallel concerns, this work may have the potential to impact both.

Unavoidable repetition occurs between chapters as a result of the chapters being published or submitted for publication as scientific papers. Repetition has been minimised where possible.

Chapter 2: Optimized extraction of anthocyanins from Reid Fruits' *Prunus avium 'Lapins'* cherries

An original version of this chapter was published in Food Chemistry journal as an original research investigation. It appears in the literature as:

Blackhall ML, Berry R, Davies NW, Walls JT. 2018. Optimized extraction of anthocyanins from Reid Fruits' *Prunus avium 'Lapins'* cherries. Food Chemistry, pp.280–285.

Thomson Reuters Journal Impact Factor: 4.946

SJR journal ranking: Q1

Rationale

This study was conducted to investigate process parameters to develop optimised method for extracting anthocyanins from cherries. The optimised protocol was developed to increase yield/ recovery of anthocyanin from waste fruit. This is the first study to scrutinise parameters of time, temperature, solvent (type and concentration) and method of breaking down fruit as central to enhancing anthocyanin recovery and yield in one study. The results from this published study have shown there is commercial viability with optimised extraction technique.

Abstract

The influence of process parameters on the extraction of anthocyanins from the edible portion of fresh, sweet cherries were investigated. The optimal extraction time and temperature were determined as 90 minutes and 37°C, respectively. A solvent/solid ratio of 10mL/g using 100% acidified solvent resulted in the greatest anthocyanin yield. No significant difference was observed between the use of methanol or ethanol as the extraction solvent. Ultra-Performance Liquid Chromatography-MS analysis of the extract identified four anthocyanins, with cyanidin-3-rutinoside and peonidin-3-rutinoside accounting for over 95% of the anthocyanin content, while cyanidin-3-glucoside and pelargonidin-3-rutinoside accounted for the remaining 5%. 244mg/100g fresh weight total anthocyanins were determined in the fresh cherries using the optimal extraction conditions.

Keywords: Anthocyanin; sweet cherry; extraction; *Prunus avium Lapins*

2.1 Introduction

Many plants contain bioactive compounds that have been shown to be effective in treating a variety of pathologies, particularly diseases associated with oxidative stress (139, 140). Polyphenolics are a class of bioactive compounds that have been a recent research focus due to their high antioxidant activity (139, 140, 144). Anthocyanins are members of a subclass of polyphenolic flavonoids and are responsible for the dark purple, blue and red colours of many fruits and vegetables. A number of studies have investigated the anthocyanin content and antioxidant activity of plant extracts, including those from blueberries, wild berries and grapes (13, 150, 151).

Cherries are known to be a rich source of anthocyanins, and the anthocyanin content

of a number of cherry varieties has been reported (appendix 2) (10, 67, 74, 77, 79, 80, 89, 91, 94, 95, 117, 152-156). The reported anthocyanin content of sweet cherry (Prunus avium) cultivars is inconsistent; for example, 26 to 225mg/100g for Prunus avium 'Bing' cherries and 13 to 165mg/100g for Prunus avium 'Burlat' cherries (72, 80, 85, 89, 154). It is unlikely that such variation can be ascribed to differences in geographical location and environment alone and are more likely explained by different post-harvest factors, including storage conditions and extraction methods.

A number of methods for the extraction of anthocyanins have been described, including pressure-liquid-, microwave- and ultrasound-assisted extraction and the traditional maceration in solvent method (111, 112, 115) as outlined in chapter 1. However, maceration is an attractive approach for smaller laboratories, as it requires no specialist equipment, but the type of solvent, solvent/solid ratio and the temperature at which the extraction is performed may affect extraction efficiency and need to be controlled. Good yield of extraction with acidified solvent has been reported in different anthocyanin rich foods, yet there is no consensus as to the most efficient combination. In strawberries, acetone: acetic acid achieved high extraction yield (116) as compared to ethanol-water:acetic acid solvent in blackcurrants (107), methanol:acetic acid in purple fleshed potatoes (110), and methanol:water in mulberry fruit (114). Optimization of extraction conditions not only results in a greater yield of the compound of interest and clarity in solvent application, but also allows a more accurate quantitative determination of the compound(s) (157). The ideal extraction conditions can vary dependent on the type of plant and its anthocyanin profile, and as such optimization of extraction from different food sources is both commercially and scientifically very valuable (109).

While optimization of the maceration method has previously been investigated in sour

cherry pomace (158) (a by-product of juice production), there are no comprehensive studies to date investigating extraction conditions for sweet cherry cultivars, nor for extraction from whole fruit. The objective of this study was to optimize the extraction of anthocyanins from the whole fruit of sweet cherries by investigating how extraction time, extraction temperature, solvent/solid ratio and solvent type influence anthocyanin yield.

2.2 Materials and methods

2.2.1 Reagents

Methanol (Merck, Germany); 12N hydrochloric acid (HCl) (Sigma Aldrich, USA); cyanidin chloride standard (Sigma Chemical Company, USA); Ethanol (Fronine, Australia).

2.2.2 Cherries

Prunus avium Lapins cherries (supplied by Reid Fruits, Plenty, Tasmania, Australia) were harvested by hand at commercial maturity stage. On the day of harvest, cherries were thoroughly washed by rolling in ice water, followed by de-stemming by hand and de-pitting using a Steinomat manual cherry stoner (Westmark, Germany). The pitted cherries were stored at -20^{o}C for 24 hours followed by immersion in liquid nitrogen and storage at -80^{o}C until extraction.

2.2.3 Preliminary extractions

Frozen cherries were thawed at room temperature followed by either bruising in a mortar and pestle or being homogenization in extraction solvent using a SilentCrusher M homogenizer (Heidolph, Germany). Cherries were broken down (via bruising or homogenization) to enable comparison before anthocyanins were extracted using the

maceration method as described by Ghassempour et al. (2008) (111). In brief, 2g of thawed pitted cherries (either bruised or homogenized) were macerated in methanol containing 0.1% 12N HCL (2 x 50mL) for 48 hours. Extraction solvent was retained, and fresh solvent added to the fruit pulp once during extraction period. After 48 hours, extracts were combined and centrifuged at 2400g for 15 minutes in an Allegra X-15R centrifuge (Beckman Coulter Inc., USA). Supernatant was retained and solvent evaporated as described in section 2.4. The remaining extract was diluted in 50mL of 0.1% HCL, followed by filtration through 0.45μM and 0.22μM syringe filters. Samples were stored at -20^{o}C prior to UPLC analysis.

2.2.4 Anthocyanin extraction

Frozen cherries were broken into small pieces in a mortar and pestle and thawed at room temperature for 5 minutes immediately prior to extraction. Approximately 3g of thawed cherry pieces were homogenized in 5mL extraction solvent using a SilentCrusher M homogenizer (Heidolph, Germany). Solvent was added to experimental volume, and sample was extracted under the conditions described in "Optimization experiments". Following extraction, samples were centrifuged at 2400g for 15 minutes in an Allegra X-15R centrifuge (Beckman Coulter Inc., USA). The supernatant was transferred to a round-bottomed flask and the solvent evaporated using a rotary evaporator (Buchi, Switzerland) at 39^{o}C. The remaining extract was diluted in 50mL of 0.1% HCL, followed by filtration through 0.45μM and 0.22μM syringe filters. Samples were stored at -20^{o}C prior to UPLC analysis.

2.2.5 Optimization experiments

Extraction temperature, extraction time, solvent/solute ratio, solvent type and solvent concentration were varied to determine their influence on anthocyanin yield. The

optimal condition for each parameter was determined according to the highest total anthocyanin content (TAC), expressed as mg cyanidin-3-glucoside equivalents/100g of fresh pitted cherry weight, and was used for all subsequent optimization experiments.

2.2.5.1 Time and temperature of extraction

Samples were extracted in methanol containing 0.1% 12N HCL at a solvent/solid ratio of 5mL of solvent per gram of fresh pitted cherry weight (5mL/g). Samples were extracted for between 30 minutes and 24 hours at temperatures ranging from 4^{o}C to 70^{o}C.

2.2.5.2 Solvent/solid ratio

Samples were extracted using the optimal time and temperature of extraction as determined in section 2.2.5.1. Samples were extracted in methanol containing 0.1% 12N HCl at a solvent/solid ratio of 2.5, 5, 7.5, 10 or 12.5mL/g of fresh pitted cherry weight. Extraction solvent was replaced twice during the extraction period.

2.2.5.3 Selection of solvent type

Samples were extracted using the optimized time, temperature and solvent/solid ratio as determined in sections 2.2.5.1 and 2.2.5.2. Samples were extracted in ethanol containing 0.1% 12N HCL or methanol containing 0.1% 12N HCL. Extraction solvent was replaced twice during the extraction period.

2.2.5.4 Solvent concentration

Samples were extracted using the optimized parameters as determined in sections 2.2.5.1, 2.2.5.2 and 2.2.5.3 at solvent concentrations of 0 to 100% (with 0% being water, and 100% being pure solvent). Extraction solvent was replaced twice during

the extraction period.

2.2.6 Anthocyanin quantitation and identification

Quantitation of total anthocyanins was accomplished by summing areas under the peaks observed at 497nm by ultra-performance liquid chromatography (UPLC) using a diode array UV/Vis detector (the detector range was only to 500nm). The detector response was calibrated using a cyanidin chloride standard (Sigma Chemical Co.) to enable conversion of peak areas into weights of cyanidin or cyanidin-3-glucoside equivalents. Targeted analytes for UPLC-MS by Multiple Reaction Monitoring (MRM) were selected on the basis of earlier in-house studies and published data for cherry anthocyanins (85).

10µL aliquots of the extracted sample were injected into a Waters Acquity H-series UPLC coupled to a Waters Acquity PDA detector in series with a Xevo triple quadrupole mass spectrometer. A Waters Acquity UPLC BEH C18 column (2.1 x 100mm x 1.7-micron particles) was used, with mobile phases A (5% formic acid) and B (acetonitrile). The column was held at 35^{o}C, the flow rate was 0.35 mL/min, with 95% A 5% B for 2 minutes, then a linear gradient to 82.5% A and 17.5% B at 7 minutes, followed by 3 minutes re-equilibration to original conditions. The PDA was monitored continuously over the range 210 to 500nm.

The mass spectrometer was operated in positive electrospray ionization mode. The ion source temperature was 130°C, the desolvation gas was nitrogen at 950 L/hr, the desolvation temperature was 450°C and the capillary voltage was 2.7KV in all cases. Targeted analytes using MRM are listed in (Table 2.1). Dwell time was 61ms per channel. The cone voltages and collision voltages for the glucosides were 35 and 25V respectively, and for the rutinosides 40 and 30V respectively.

Table 2.1 UPLC parameters used for identification of anthocyanins from *Lapins* cherries.

Anthocyanin	Precursor ion	Product ion
pelargonidin-3-glucoside	433.10	271.05
cyanidin-3-glucoside	449.10	287.05
peonidin-3-glucoside	463.10	301.05
delphinidin-3-glucoside	465.10	303.05
petunidin-3-glucoside	479.10	317.05
malvidin-3-glucoside	493.10	331.10
pelargonidin-3-rutinoside	579.20	271.05
cyanidin-3-rutinoside	595.20	287.05
peonidin-3-rutinoside	609.20	301.05
delphinidin-3-rutinoside	611.20	303.05
petunidin-3-rutinoside	625.20	317.05
malvidin-3-rutinoside	639.20	331.10

2.2.7 Statistical analysis

Experimental results were analysed using Prism statistical software Version 6.0 (GraphPad Software, Inc., USA). Results are reported as the mean of at least three replicates, identically handled and using the same samples taken from the harvested sample as described in section 2.2, ± standard error. One- or two-way analysis of variance (ANOVA) with Tukey's correction for multiple comparisons was used to determine significant differences (p-value < 0.05) between the means.

2.3 Results and discussion

2.3.1 Identification of anthocyanins present in the extracts

Individual anthocyanins were separated via UPLC, with peak assignment based on matching UV-visible spectra with retention times from previous in-house studies and published cherry anthocyanins (85). Four different anthocyanins were detected in all extracts (Figure 2.1). The major anthocyanin identified was cyanidin-3-rutinoside and accounted for approximately 80% of anthocyanin equivalents in all samples. Peonidin-3-rutinoside was identified as the second major pigment, accounting for approximately 15% of anthocyanin equivalents, whilst two minor peaks, identified as cyanidin-3-glucoside and pelargonidin-3-rutinoside, accounted for the remaining 5% of detected anthocyanin equivalents.

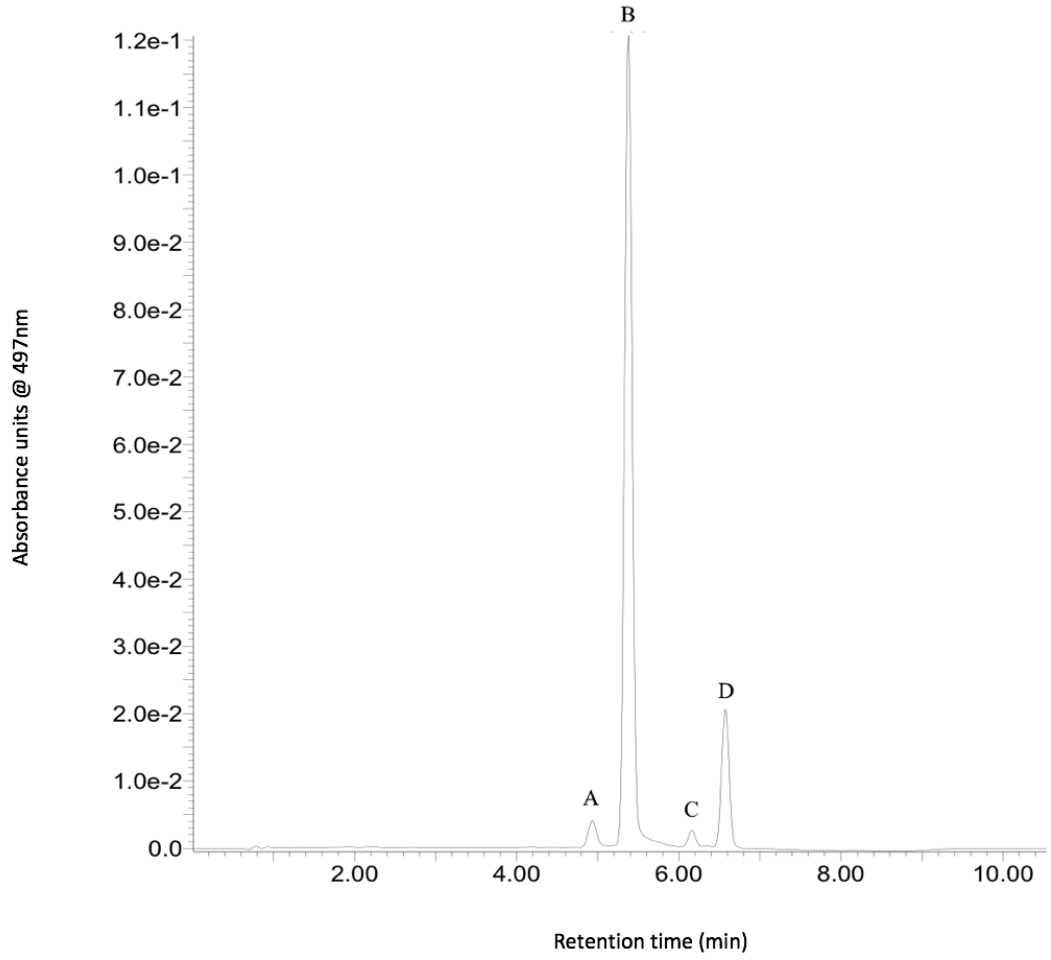


Figure 2.1 UPLC chromatogram of anthocyanin profile of *Lapins* cherries registered at 497nm.

Reference peaks on the chromatogram represent the following compounds: A-cyanidin-3-glucoside; B – cyanidin-3-rutinoside; C – pelargonidin-3-rutinoside; D – peonidin-3-rutinoside.

Whilst cyanidin-3-rutinoside has previously been identified as the major anthocyanin in sweet cherry cultivars, the identification of peonidin-3-rutinoside as a major pigment in *Lapins* cherries is unique. Peonidin-3-rutinoside has previously been found to make only a minor contribution to overall anthocyanin content, with a number of studies identifying cyanidin-3-glucoside as the second major pigment (85, 89, 156). Serra et al. (2011) and Usenik et al. (2008) investigated the anthocyanin profile of a variety of sweet cherry cultivars in Portugal and Slovenia, respectively. For both studies,

peonidin-3-rutinoside accounted for less than 2% of total anthocyanin content in *Lapins* cherries (85, 156). Geographical location and environmental factors, including temperature, water status and light exposure, have been shown to impact anthocyanin content, therefore the conflicting results found in this study may be partly attributable to the contrasting environments in which the cherries were cultivated (69, 159, 160).

2.3.2 Optimization of experimental conditions

2.3.2.1 Preliminary extractions

Extraction for 48 hours utilizing bruised cherries resulted in a mean anthocyanin yield of 74.99 ± 9.83mg/100g of fresh pitted cherry weight (data not shown). This was lower than the previously reported anthocyanin concentrations of sweet cherries (72, 80, 85, 89, 154) and it was hypothesized that bruising did not cause adequate disruption of the fruit matrix to allow efficient extraction. Therefore, a subsequent extraction in which the fruit was homogenized was performed. Homogenization resulted in a significant increase ($p=0.009$) in anthocyanin yield to 133 ± 7.12mg/100g of fresh pitted cherry weight, and as such all subsequent optimization experiments were carried out using homogenized cherries.

2.3.2.2 Extraction time and temperature

Previous research has indicated that increased temperature leads to improved solid-liquid phase extraction efficiency, due to enhanced diffusion rate and analyte solubility (161). However, elevated temperatures have also been suggested to increase the rate of anthocyanin degradation (162-164). Time and temperature were investigated simultaneously to reveal any interaction between the two parameters and allow determination of the optimal conditions for anthocyanin extraction. Cherries were extracted for between 30 minutes and 24 hours at 4, 22, 37, 52 and 70^{o}C.

The highest anthocyanin yield was observed following extraction at 37°C (Figure 2.2). TAC reached 222.82 ± 8.88mg/100g of fresh pitted cherry weight at 1.5 hours, after which further increases in extraction time resulted in a decrease in yield. Samples extracted for 1.5 hours at 37°C had significantly higher anthocyanin content than those extracted for 1.5 hours at 22°C ($p < 0.005$), 52°C ($p < 0.05$) or 70°C ($p < 0.001$) (Figure 2.2). Anthocyanin content was also lower in the 4°C samples, although not significantly. These results are in agreement with previous research by Cacace and Mazza (2002) investigating anthocyanin extraction from whole blackcurrants, who found that anthocyanin yield increased with temperature up to 35°C, whilst further temperature increases resulted in a decrease in yield (107). In contrast, temperatures as high as 93°C and 75°C have been reported as optimal in studies investigating extraction from grape and sour cherry pomace, respectively (158, 165). These differences may be attributable to the pomace having already been exposed to high temperatures during processing, and therefore heat sensitive anthocyanins may already have been degraded.

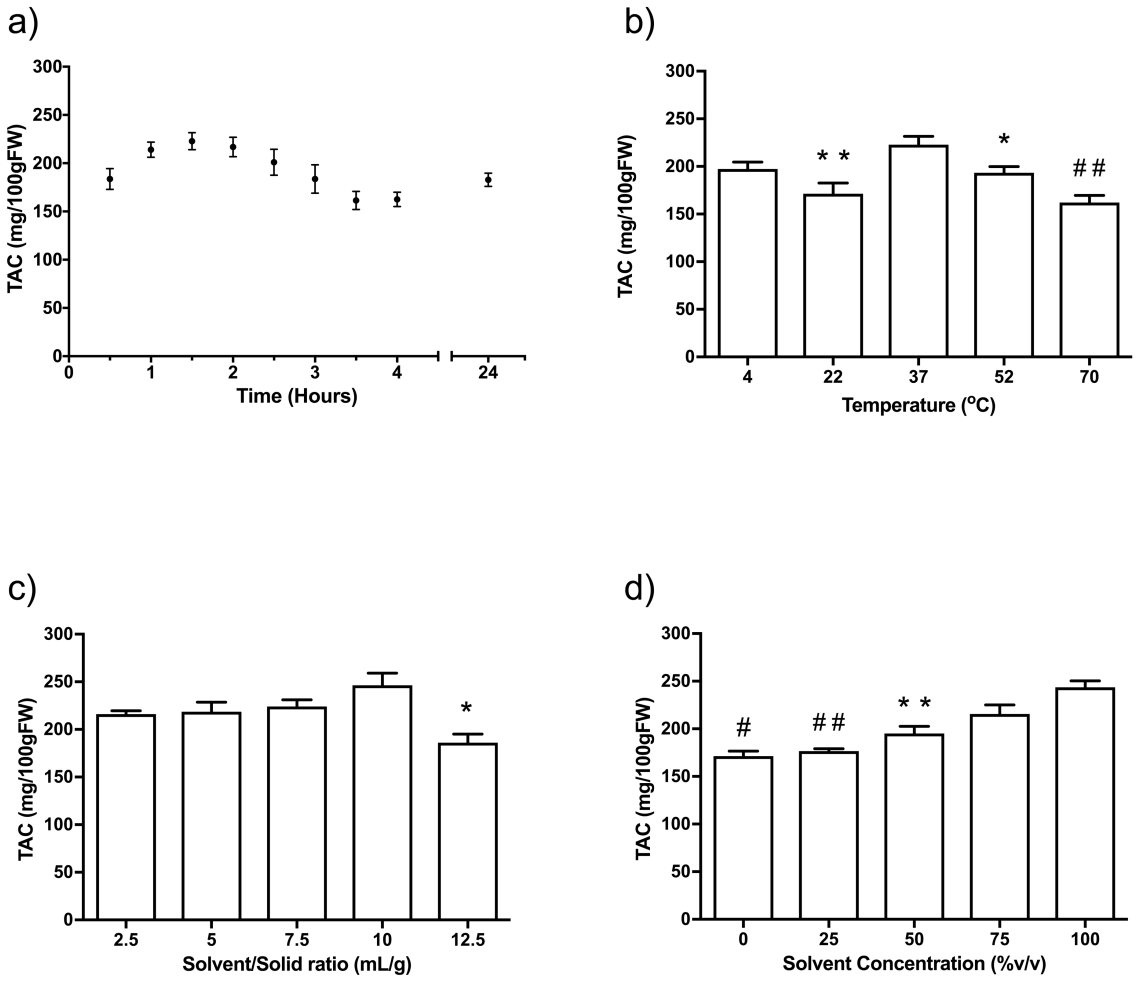


Figure 2.2 Effect of a) time, b) temperature, c) solvent/solid ratio and d) solvent concentration on the extraction of anthocyanins from fresh sweet *Lapins* cherries.

TAC – total anthocyanin content determined via UPLC. * - p<0.05, ** - p<0.01, # - p<0.005, ## - p<0.001 Error bars represent standard error of the mean for at least 3 replicates.

2.3.2.3 Effect of solvent/solid ratio on anthocyanin yield

Determining the optimal solvent/solid (S/S) ratio is important when designing extraction protocols not only to ensure efficient extraction of the desired compound, but also to reduce extraction costs, which are of particular importance when performing large-scale extractions. The S/S ratios investigated in this study were based on previous

research into the extraction of anthocyanins from sources such as blackcurrants, rose myrtle fruit skin and sour cherry pomace (33, 107, 158). Samples were extracted for 90 minutes at 37^{o}C at solvent/solid ratios varying from 2.5 to 12.5mL/g.

The effect of the S/S ratio on the extraction of anthocyanins followed mass transfer principles and Fick's law of diffusion (Figure 2.2). Anthocyanin yield increased with increasing S/S ratio from 2.5 to 10mL/g, due to the corresponding increase in concentration gradient and the amount of solute that was able to come into contact with the solvent. A further increase in S/S ratio to 12.5mL/g resulted in a significant decrease in TAC ($p < 0.01$). The optimal S/S ratio for anthocyanin extraction has been shown to vary dependent on a number of factors, including the source material and how the material is processed prior to extraction (85, 89, 156, 158). Yilmaz et al. (2015) determined the optimal S/S ratio for anthocyanin extraction from sour cherry pomace to be 12.5mL/g. The slightly lower optimal S/S ratio found in this study (10mL/g) is most likely due to the use of whole fruit rather than dry pomace.

2.3.2.4 Solvent type

Methanol and ethanol are the most common solvents used to extract anthocyanins. Previous research indicates that acidification of the solvent leads to a greater anthocyanin yield (166, 167). The addition of acid to the extraction solvent can increase anthocyanin extraction efficiency in two ways; the acidic pH denatures cell membranes, allowing easier interaction between the solvent and the anthocyanins, and the free hydrogen ions stabilize the coloured flavylium cation form of the anthocyanin (168). The current study compared the extraction efficacy of acidified methanol and acidified ethanol (both containing 0.1%v/v 12N HCL).

Extraction with acidified methanol resulted in the highest anthocyanin yield (249 ±

4.48mg/100gFW), however acidified ethanol was still able to recover 94% of the TAC. These results are in agreement with studies investigating anthocyanin extraction from other sources, and are likely due to the slightly higher polarity of methanol as compared to ethanol (166, 169). Ethanol is generally recognized as safe (GRAS) for human consumption and is often preferred to methanol as an extraction solvent, particularly in relation to the extraction of anthocyanins designed for food applications. Due to its GRAS status and results indicating no significant reduction in anthocyanin yield, further optimization experiments were undertaken using acidified ethanol as the extraction solvent.

2.3.2.5 Solvent concentration

Results indicated a positive relationship between solvent concentration and anthocyanin yield, with TAC being highest in samples extracted with pure acidified ethanol (244mg/100gFW, Figure 2.2d). This yield was slightly (but not significantly, $p > 0.10$) higher than samples extracted with 75% solvent, and significantly ($p < 0.01$) higher than yields achieved using 50% solvent, 25% solvent or acidified water. Previous research has suggested that aqueous solvents are more effective at extracting anthocyanins than pure solvents - an ethanol concentration of 69 to 70% has been shown to be most effective at extracting anthocyanins from grape pomace (165, 170), whilst 44% ethanol has been indicated as the optimum concentration for extraction from sour cherry pomace (158). The conflicting results found in this study may partially be attributable to the difference in the matrices of the anthocyanin source. The water content of fresh sweet cherries has been estimated to account for 81% of the edible fruit weight (171), and would have contributed to a reduction in the final concentration of the solvent.

2.4 Conclusions

An optimized extraction procedure was determined for the extraction of anthocyanins from fresh, whole sweet cherries (*Lapins* cultivar). The optimal extraction parameters were a 90-minute extraction time, 37°C temperature, 10mL/g solvent to solid ratio and 100% acidified ethanol concentration. To the best of our knowledge, this is the first study to comprehensively investigate the effects of time, temperature, S/S ratio, solvent type and solvent concentration on the extraction of anthocyanins from fresh, whole sweet cherries. This information will be valuable from both a commercial and scientific perspective in its own right but also to enable the quantities of compound needed for functional experiments to be obtained more easily.

2.5 Acknowledgments

Associate Professor John Ross, School of Plant Science, University of Tasmania, for technical expertise and use of laboratory facilities. Dr David Nichols, Central Science Laboratory, University of Tasmania, for technical assistance with UPLC.

This work was funded by the Australian Department of Industry & Science and Essential Oils of Tasmania. No funding bodies had any involvement in the conductance of this study.

Figure 2.3 Photo of cherry extract on completion of extraction steps

Chapter 3: Anthocyanin content of sweet cherries: differences in fruit grade, cultivar, seasonal variation and storage conditions

Rationale

This study was conducted to determine if there was variation between fruit grade, cultivar and season. It was also an additional reliability measure to determine if the fruit that was stored post-harvest, degrades under different conditions. This study provides much needed context to the value and potential use of waste fruit and will inform post-harvest storage of fruit to ensure anthocyanin is not lost.

Abstract

Although approximately 18,500 tonnes of sweet cherries (Prunus avium L.) are produced annually in Australia, an estimated 20 to 50% of each season's crop is deemed unfit for market. Cherries are known to be a rich source of anthocyanins so the recovery and conversion of this waste into value-added products has potential for economic and environmental benefits. However, whether the anthocyanin content of Australian sweet cherries is influenced by season, storage conditions or cultivar type is not known. The aim of this research was to ascertain the difference in total anthocyanin content (TAC) of waste compared with premium grade fruit and to determine if season, storage and cultivar type affect TAC. Premium and waste cherries were harvested at commercial maturity across three consecutive growing seasons from 2014 – 2017 and after grading, were stored at either -20^{o}C or -80^{o}C. At 0, 3, 6, 12- and 24-months post-harvest, anthocyanins were extracted and the anthocyanin profile and total anthocyanin content (TAC) were determined via ultra-performance liquid chromatography UPLC. Waste fruit was up to 43% higher than

premium grade fruit and significant differences in total anthocyanin content were identified between varieties. Kordia (Prunus avium L. 'Kordia') was the richest source of anthocyanins, with a TAC of 873mg/100g, followed by Vans (Prunus avium L. 'Vans'), Sweet Georgia (Prunus avium L. 'Sweet Georgia'), Simone (Prunus avium L. 'Simone') and Lapins (Prunus avium L. 'Lapin'). There was also a significant difference in TAC between fruit stored at -20°C and -80°C, with as little as 4.4% TAC remaining after 24 months at -20°C. This suggests that the currently under-utilised cherry waste fruit provides a potential economic benefit for producers, with high amounts of valuable TAC for extraction. Furthermore, the Kordia (Prunus avium L. 'Kordia') cultivar warrants further study due to its high TAC.

Key words: Anthocyanin; sweet cherries; Prunus avium L.' Kordia'; waste fruit

3.1 Introduction

Fruit and vegetable waste products have traditionally been used as feed (172), silage for livestock (15), compost (14), biofuel (173, 174) and more recently as lignocellulosic biomass (LCB) (12, 175, 176). The conversion of LCB waste material into renewable energy has been heralded as a solution for the disposal of plant waste by significantly reducing the reliance on landfill (176-178). However, the diversion of these agricultural wastes into human foods is more desirable, with the potential to deliver a greater economic return (179, 180).

Research into developing waste food into a commercial product has focussed on foods with high concentrations of bioactive phenolic compounds (179), such as anthocyanins. Currently, anthocyanins from elderberry, red beet, hibiscus and red cabbage are used in the food industry as a natural colourant (181, 182). However,

due to their many health benefits, an additional focus has been their potential use as a nutraceutical. Recent studies suggest that anthocyanin-rich compounds may improve digestion (183), be neuroprotective (184) and improve skin hydration when applied topically (185).

Sweet (Prunus avium L.) and sour (Prunus cerasus) cherry varieties provide a rich source of anthocyanins (73, 94, 153, 186), with global production estimated to be in excess of 3.7 million tonnes annually (http://www.fao.org/faostat/en/#data/QC). In Australia, sweet cherry cultivars are preferentially grown, with 18,500 tonnes produced annually (118). Whilst production is small on a global scale, the crop is a valuable commodity contributing $164 million to the local economy in 2016 (118). The temperate climate of southern Australia provides ideal growing conditions (119), as demonstrated by the Southern state of Tasmania, producing cherries at a rate of 7.1 tonnes per hectare, a 25% greater yield than any other region in Australia (118). However historically, 20-50% of the crop is deemed unfit for market where the majority of this fruit is dumped with only a small proportion being re-purposed (120). Identification of methods that efficiently transform this waste into commercially viable products presents an opportunity for the industry. To support this research, a detailed understanding of the type(s) and potential yield of anthocyanins from waste fruit is needed.

The anthocyanin profile and concentration found in the fruit varies significantly between cherry varieties (85, 89, 152, 156). In Australia, there are over 80 varieties grown, with 20 varieties contributing to commercial output (119) yet limited research into the individual cultivars and their bioactive compounds has been completed. The majority of research into cherry anthocyanin content has been undertaken in premium grade fruit and sour varieties (59, 187), with minimal studies examining waste fruit or sweet

cherry cultivars. Research in sour cherry cultivars has focussed on profiling anthocyanin content, determining antioxidant capacity (10, 149, 153), identification of anti-inflammatory properties (188), determining impact on melatonin regulation and sleep quality (189) and examining the influence on exercise performance and recovery (190-193).

As a comprehensive characterisation of the anthocyanin profile of a range of sweet cherry cultivars is lacking, particularly those identified as having commercial significance, research on this topic is warranted. The aims of the present study were to profile the anthocyanin content of premium grade and waste fruit from a range of sweet cherry cultivars across different growing seasons.

3.2 Materials and Methods

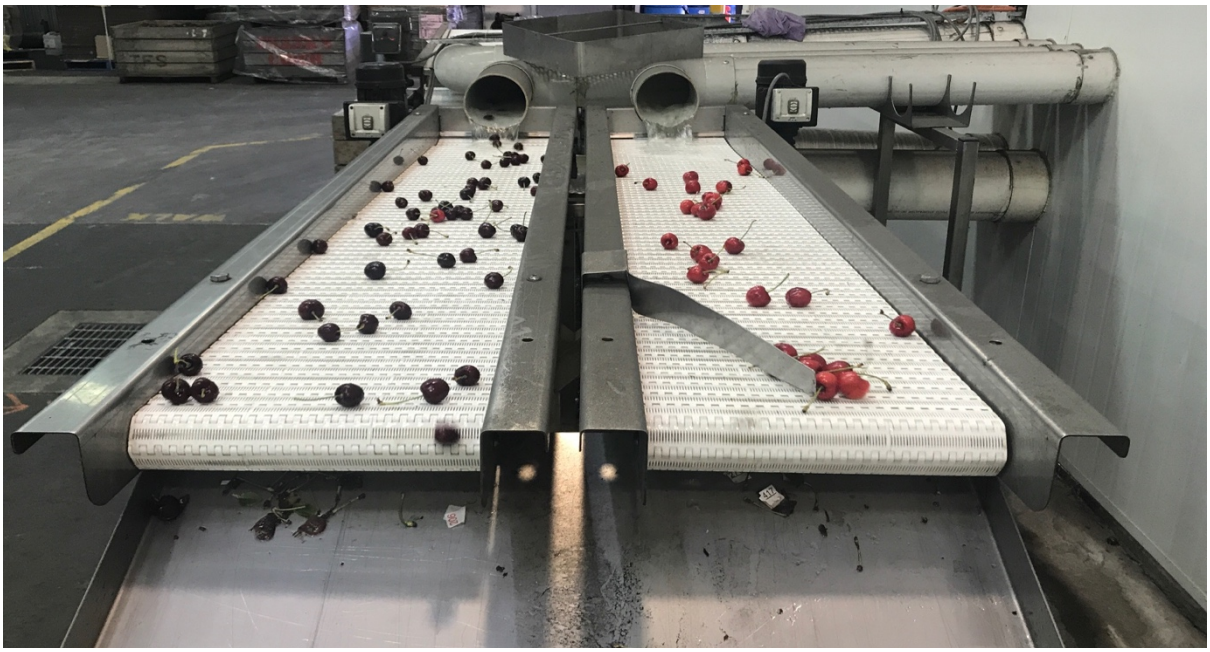
3.2.1 Cherry sourcing

Fresh Prunus avium L. cherries that had been graded according to industry standards were sourced from Reid Fruits (Plenty, Tasmania, AUS). The cultivars investigated in this study were Lapins, Kordia, Regina, Simone, Sella, Vans, Sylvia, Sweet Georgia, Fertard and Sweetheart (Prunus avium L.). For commercial reasons, not all varieties were available each year, as such sampling was opportunistic based on availability.

3.2.2 Cherry selection

In brief, after being hand-picked, the fruit underwent a two-step grading process to separate the fruit into first (premium), second and third grade (waste). Firstly, it was graded (Airjet, GP Graders, Victoria, AUS) and then optically sorted according to size and colour (TrueSort, Ellips, Eindhoven, NL) figure 3.1. The optical grading used high intensity LED lighting to illuminate the fruit, and then it was photographed using high

definition cameras, which enabled the highest recovery of premium grade fruit (appendix 3). The third grade or waste fruit was separated out and stored for disposal.



Sorting and separating fruit according to colour.

Cherries sorted according to colour and deemed waste fruit.

Figure 3.1 Cherry processing showing sorting according to colour.

The imperfections that would deem the fruit unsuitable for the fresh fruit market and categorise it as first, second or third grade include splits, cracks, pits, softness and colour variation (194) figure 3.2.

Waste: splits and cracks Waste: small in size

Figure 3.2 Examples of characteristics that deem fruits premium grade or waste fruit.

Samples of the premium and waste fruit were collected at maturity in 2014/2015, 2015/2016 and 2016/2017 seasons. After the cherries were transported to the laboratory they were washed and de-stemmed by hand, and then de-pitted using a Steinomat hand processer (Westmark, Lennestadr-Elspe, GER). The cherries were then stored according to the experimental protocol (detail below) until processing occurred.

3.2.3 Storage Conditions

To determine the influence of storage conditions on TAC over time, premium grade Prunus avium L. ‘Lapin’ cherries were randomly allocated to either -20^{o}C or -80^{o}C storage temperatures after being refrigerated for 24 hours. Fruit from each temperature allocation was tested for anthocyanin content after the 24-hour refrigeration, and then at 3, 6, 12 and 24 months.

3.2.4 Extraction and quantitation of sweet cherry anthocyanins

The edible portion of thawed sweet cherry fruit (100g) was homogenized in acidified ethanol (1L 0.1% 12N HCL ethanol) and incubated for 90 minutes at 37^{o}C. The sample was vacuum-filtered, and the solvent evaporated to 25 mL using a rotary evaporator (Buchi, CHE) at 39^{o}C before being filtered through 45 µM and then 20 µM syringe filters. Samples were stored at -80^{o}C before being assayed by ultra-performance liquid chromatography (UPLC) and all measures were undertaken in triplicate. Quantitation of total anthocyanin content (TAC) was undertaken by the method previously reported by this group (186). In brief, quantitation was accomplished by summing areas under the peaks observed at 497nm by UPLC using a diode array UV/Vis detector. The detector response was calibrated using a cyanidin chloride standard (Sigma Chemical Co., USA) to enable conversion of peak areas into weights of cyanidin or cyanidin-3-glucoside equivalents (21). All samples were tested in triplicate to ensure reliability of measurements.

3.2.5 Data analysis

Experimental results were analysed using Prism Version 6.0 (GraphPad Software, Inc, USA). Results are reported as the mean of three replicates $\pm$ standard error. One- or two-way mixed analysis of variance (ANOVA) with Tukey's correction for multiple comparisons was used to determine significant (p-value < 0.05) differences between the means.

3.3 Results

3.3.1 Total anthocyanin content (TAC) of waste and premium grade fruit

The TAC of waste Prunus avium L. 'Lapin' cherries from the 2014/15 season was found to be 43% higher than that of premium cherries (Figure 3.3). To examine this effect further, a different cultivar was chosen from the 2016/17 growing season as a point of comparison. The TAC for Prunus avium L. 'Kordia' waste fruit in 2016/17 was 18% higher than premium grade fruit (Figure 3.3).

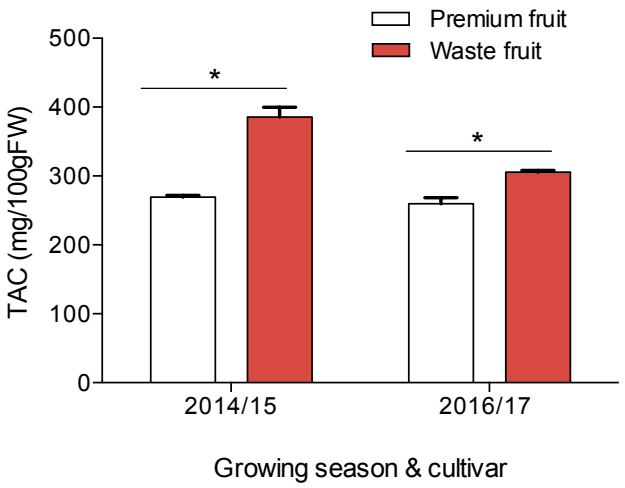


Figure 3.3 Total anthocyanin content of premium grade and waste fruit for Prunus avium L. 'Lapin' cultivar (2014/15) and Prunus avium L. 'Kordia' (2016/17) are expressed as mean ± SEM (*p < 0.01).

3.3.2 Variation of total anthocyanin content across cultivars

Across three consecutive growing seasons, 2014/15, 2015/16 and 2016/17, the anthocyanin content of premium grade fruit in a range of different cultivars was determined. Significant differences were found, both within season between the varieties, and between seasons, with TAC ranging from 138.7 to 872.9mg/100g FW (Figure(s) 3.4a, b and c). Whilst all the varieties were not available each year, Prunus

avium L. ‘Simone’ and Prunus avium L. ‘Kordia’ were consistently available. Interestingly, Prunus avium L. ‘Kordia’ cultivar had significantly higher TAC than all the other cultivars across each of the growing seasons.

The 2015/16 growing season had higher TAC in all the varieties measured compared with 2014/15 and 2016/17 (Figure 3.4). The Prunus avium L. ‘Stella’, Prunus avium L. ‘Sylvia’, Prunus avium L. ‘Lapin’ and Prunus avium L. ‘Simone’ varieties had the lowest TAC (below 400mg/100g FW) whereas the Prunus avium L. ‘Vans’ and Prunus avium L. ‘Sweet Georgia’ varieties had TACs in excess of 500mg/100g FW (Figure 3.4b). In 2015/16 Prunus avium L. ‘Kordia’ had a TAC almost three times higher than Prunus avium L. ‘Sylvia’ at 293mg/100g FW (Figure 3.4b). Compared to the average of all other varieties of that season, Prunus avium L. ‘Kordia’ had ~1.4 times the TAC.

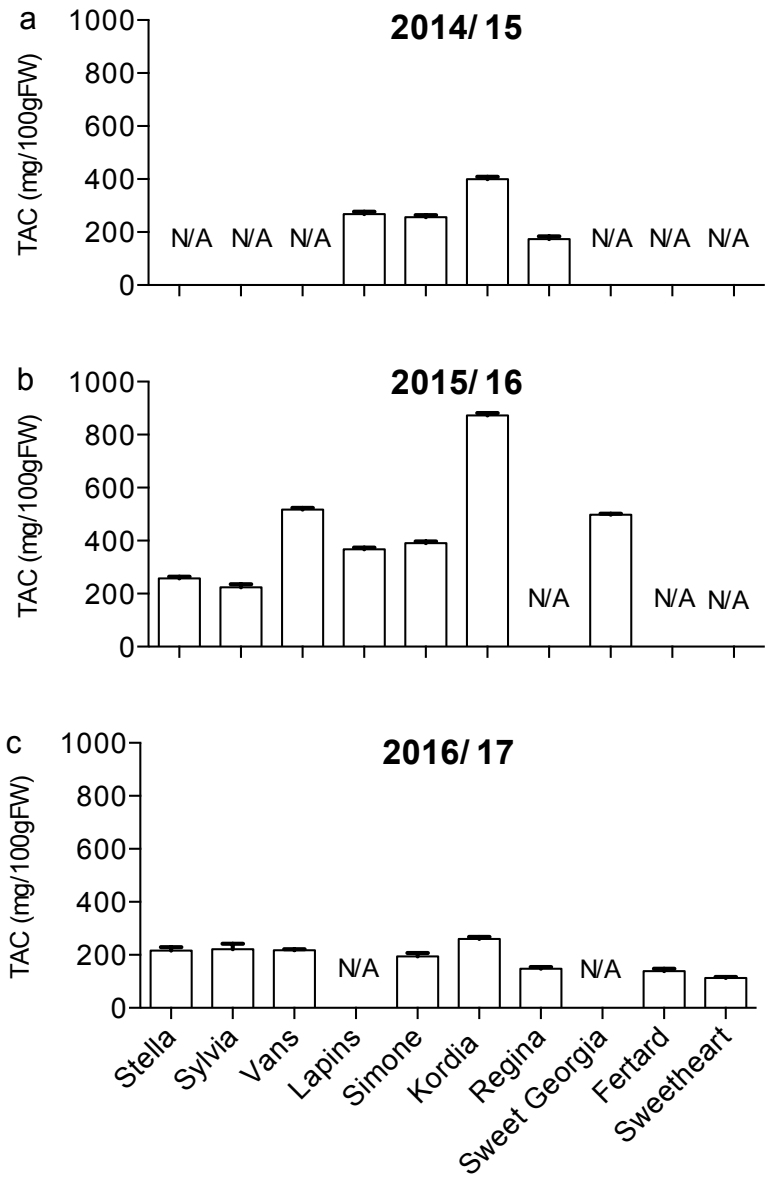


Figure 3.4 Total anthocyanin content of premium grade fruit was measured across three consecutive growing seasons (a) 2014/15, (b) 2015/16 and (c) 2016/17.

N/A represents when fruit was unavailable for testing. All samples were tested in triplicate and results are expressed as mean ± SEM with a one-way ANOVA.

3.3.3 Effect of storage conditions on total anthocyanin content

Premium grade *Lapins* were analysed over the course of 24 months to determine the effect of storage conditions on TAC. Storage at -80^{o}C for up to 12 months resulted in

a significantly higher (p=0.001) retention of anthocyanins as compared to storage at -20°C (Figure 3.5). There was a decline in the TAC of cherries stored at -20°C over 24 months. In contrast, TAC of cherries stored at -80°C decreased by only 27% following 3 months of storage, followed by a return to 99% of fresh TAC at the 12-month time point. After 24 months of storage, TAC was reduced to 10.9% and 4.4% of original levels after storage for 24 months at -80°C and -20°C, respectively (Figure 3.5).

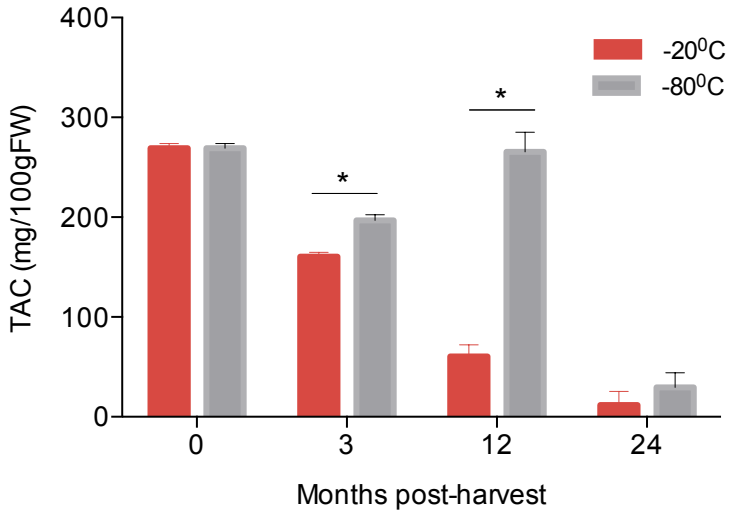


Figure 3.5 The TAC of premium cherries, stored at -20°C and -80°C, was analysed at four time-points across 24 months to determine impact of storage temperatures on post-harvest TAC. Results are expressed as mean ± SEM (*p=0.0001).

3.4 Discussion

This study yielded four noteworthy results. Firstly, cherry waste was found to be up to 40% higher in anthocyanins than premium grade fruit as observed in Prunus avium L. 'Lapin' and Prunus avium L. 'Kordia' cultivars. Thus, cherry waste has the potential to be turned into a valuable resource as has been the case with other fruit waste (12, 173, 174, 179). Aslam and colleagues (12) were able to utilise mango waste to enhance both fibre and phenolic content of biscuits. Extraction from red prickly pear

peel (waste) enabled recovery of natural colourant to be utilised in value added products providing a direct economic outcome (195). To be able to effectively re-purpose waste fruit, it is essential to understand the anthocyanin content across different cultivars and to determine what variation occurs within the fruit and between seasons.

The second finding was the significant variation of anthocyanin levels between different cultivars within the same season (intra-season variability). Of the varieties analysed, Prunus avium L. 'Kordia', had significantly higher anthocyanin than all others tested. Prunus avium L. 'Kordia' is harvested mid-late season, and only accounts for a small percentage of the overall yield of sweet cherries grown due to variable cropping (196). It is reportedly more challenging to grow, compared to other varieties, as it is difficult to set fruit and it requires more 'chilling hours' (~1300 hours) (81). The flowers of Prunus avium L. 'Kordia' are also very susceptible to frosts which adds to the challenge in production, yet if the flowers remain undamaged, the fruit thrives in cooler climates (119). This variety-specific anthocyanin content will provide useful context for primary producers and inform which varieties to plant. It may also potentially explain the higher anthocyanin content of Prunus avium L. 'Kordia', when grown in colder temperate climates like Tasmania, Australia.

The third finding of significance is the inter-season variability of TAC. For two varieties studied across three growing seasons, Prunus avium L. 'Kordia' and Prunus avium L. 'Simone', significant fluctuation of TAC was observed. The 2015/16 growing season remained the superior season for TAC of the three measured in contrast to the 2016/17 growing season, which had the lowest TAC. As a potential explanation for this variation, we retrospectively examined weather information for the region as provided by the Bureau of Meteorology (www.bom.gov.au). We identified patterns of rainfall

and temperature that appear to impact TAC (appendix 4). The highest anthocyanin levels, found in 2015/16 season, appeared to result from low rainfall during the blossoming period (August to October) and during harvesting (December to January). In addition to rainfall, temperature appeared to play an important role in producing high TAC fruit. Low temperatures during winter (winter chilling), temperatures above 13^{o}C during spring (September to October), plus higher temperatures in summer fruiting (picking) season appear beneficial. In 2016/17, there was low rainfall during the blossoming period (July), high rainfall in September and the average minimum temperature for June and July was not as cold and the low temperature is required to get a good “cold set” chill to set the fruit. Furthermore, the average maximum temperatures were not as high in 2016/17 as in the 2015/16 season. Whilst this information is retrospective only, it does potentially identify the ‘ideal’ weather conditions for a high TAC crop. The possibility of predicting crop losses/gains due to weather patterns is worthy of further exploration and would be paramount for producers to plan for favourable season outcomes.

Finally, it was evident that storage temperature and storage time both significantly impact the rate of degradation of anthocyanins. The novelty of this work was our ability to demonstrate the precipitous decline in TAC at 24 months post-harvest at either -20^{o}C or -80^{o}C. Our findings are in line with previous work into polyphenol degradation in elderberry, raspberry and blackberry juice after storage at -20^{o}C but for short time periods of up to 12 months (125, 197, 198). Conversely, work by Hager et al (126) found no impact of storage at -20^{o}C for blackberry fruit, although the storage period was only 6 months, and if measured for a longer time period may have been in line with our findings.

Of additional interest in this study was that cherries stored at -80^{o}C showed a

significant increase in TAC at the 12 month time period, a result in line with research by Allaith and colleagues (199) who also found freezing increased TAC in dates. Conversely work by Lohachoompol and colleagues (66) found freeze-drying blueberries caused a decrease in overall anthocyanin. However, the fruit was only tested after 6 months, so potentially if analysed again at 12 months this result may have been in line with our findings. Conversely, it is possible that freezing for longer periods alters the concentration through osmotic dehydration (200, 201). It is expected that on freezing anthocyanins release occurs due to ice crystal formation under the skin of the fruit, causing the membrane to rupture and release the anthocyanins (202). To account for this, the cherries were frozen for 24 hours at -20^{o}C, before lowering them into liquid nitrogen which would reduce the drying effect. Chromatograms at each of the storage timepoints (0, 3, 12, 24 months post-harvest appendices 5a-d) were scrutinised to provide reasoning as to the increase in anthocyanin recorded at 12 months storage at -80^{o}C. There was no difference in the anthocyanins observed at 12 months storage, the only difference in anthocyanins was at 24 months post-harvest at -20^{o}C whereby there was an additional peak observed (appendix 5d). This increase in anthocyanins determined at -80^{o}C after 12 months storage and the additional peak identified at 24 months storage at -20^{o}C could indicate a degradation of the side chains. Whereby the samples at -20^{o}C are most susceptible to degradation which is why there is precipitous decline in the anthocyanins, due to the cell membrane rupture from ice crystal formation. At -80^{o}C over the first 12 months storage there is more release of anthocyanins, albeit passive, until all the anthocyanin is released after which it degrades (as can be seen at 24 months).

Consequently, if the waste cherry fruit was to be used for extraction, freezing at minus 80^{o}C would be an acceptable method, with 12 months storage being optimum for TAC.

There is currently some debate that the state of ripeness/colour that the fruit holds prior to being stored impacts the rate of degradation (203). Whilst measures of colour were not analysed in this work, this issue warrants further study, and could provide reasoning as to the high levels of TAC in waste fruit (due to being over-ripe or dark in colour).

3.5 Conclusion

The results of this work on cherry TAC suggest there is value in the underutilised waste material, whether it be nutraceuticals, or other products. This would allow producers to turn waste product into an economically viable commodity. The retrospective examination of weather patterns and impact on TAC also warrants further study, as it could be useful to allow growers to forecast and prepare for waste fruit. Whilst storage does impact the rate of degradation of the bioactive components, consideration as to the time and temperature under which fruit is stored, is advisable if the fruit is to be for extracting anthocyanins. From this body of work, it is evident that increased understanding functional stability is imperative when considering the potential application and extraction of bioactive compounds. Future studies should address the effect of geographical location on anthocyanin yield.

Chapter 4: Sweet cherry anthocyanins reduce weight gain and inflammation in high fat fed mice

RATIONALE

This study was conducted to investigate the functional capacity of sweet cherry derived anthocyanins in vitro and in vivo. As a progression from findings in chapters 2 and 3 it was important to gain an understanding of capacity of the extract to attenuate disease or to change metabolic process at a cellular level and then in a whole system.

Abstract

Obesity and associated inflammation are major risk factors for cardiovascular disease, type 2 diabetes and some cancers, making obesity a global health concern. Since drugs used to treat obesity elicit side effects, identifying non-drug treatments are worthwhile. The aim of this study was to assess the efficacy of sweet cherry anthocyanins (SCA) as a treatment and preventative therapy for obesity and inflammation. LPS-stimulated RAW267.4 macrophages were incubated with SCA to determine effectiveness of reducing inflammation in vitro. Treatment with SCA significantly reduced secretion of IL10 by 69.6% ($p=0.02$) and GM-CSF by 46% ($p<0.001$) relative to untreated cells. High fat fed C57BL/6J mice provided the in vivo context with the SCA tested in both prevention and reversal arms of a supplementation trial. Mice in the prevention trial gained 19% less weight ($P<0.05$) following 6 weeks supplementation with SCA (40mg/kg BW/ day). No significant difference in weight was found in the 10-week reversal arm of the trial yet SCA supplementation did cause

reduced inflammation in the mice. Therefore, as SCA significantly reduced the rate of weight gain and the associated chronic low-grade inflammation induced by a high fat diet, they provide a potential non-drug therapy for obesity and inflammation.

Key words: Anthocyanins; sweet cherry; obesity; inflammation

4.1 Introduction

High fat, high calorie diets and resultant chronic low-grade inflammation in obesity have been identified as precursors to the development of disease states such as coronary artery plaque formation and insulin resistance (30, 204-207). As the global burden of disease resulting from obesity and inflammation increases (208), there has been a focus on identifying foods that have functional properties that may reduce inflammation and associated disease (30, 31, 44, 49, 61).

Whilst there are drug strategies and treatments currently available to treat obesity (e.g. statins, appetite suppressants, pancreatic lipase inhibitors), their use often causes undesirable side effects (134, 209-211). These include nausea and vomiting, altered bowel habits, gut malabsorption syndromes and increased psychiatric events, all of which may contribute to low compliance rates (18, 134, 210). Recent studies have shown that the natural phytochemicals present in foods, including anthocyanins, might play a role in attenuating obesity by decreasing weight gain and adipose tissue without significant side effects (26, 212). However, there is a paucity of research investigating the impact of anthocyanins on obesity-associated inflammation.

Anthocyanins are water-soluble polyphenols, which give fruit, vegetables and plants their red, blue and purple hue. To date, both in vitro and in vivo studies have investigated the bioactive potential of anthocyanins, yet work connecting these

elements, in order to better understand the biological activity and mechanisms responsible is warranted. Inflammation is a complex process and RAW264.7 macrophages are universally accepted as a screen for assessing anti-inflammatory effect of bioactive substances (28, 31, 34, 213). In vivo anthocyanin research has been undertaken in both humans and animals, investigating the effect of dietary anthocyanins on cardiovascular disease, metabolic syndrome, inflammation, and insulin sensitivity (29, 30, 32, 35). Recent studies suggest that anthocyanins reduce weight gain in mouse and rat models of obesity (47, 49, 212). An unanswered question is whether anthocyanins can both prevent additional weight gain and/or reverse obesity.

Whilst research into the biological effect of anthocyanins derived from a range of sources has been carried out, the majority of research has been undertaken on the bioactive components isolated from tart cherry cultivars and other berry fruits (30, 48, 51, 214-217). The active constituents in tart cherries are cyanidin and its glycosides, primarily cyanidin-3- O-glucosylrutinoside and cyanidin-3-O-rutinoside (94), whereas in berry fruits, the primary anthocyanins are pelargonidin and its glycosides (32). Sweet cherry cultivars also contain cyanidin (85) with peonidin-3-rutinoside the major pigment in Lapin sweet cherries (186). As previous work indicates, sweet cherry varieties have significantly higher levels of these anthocyanins than tart cherries, they form the focus of the current study.

The mechanism(s) by which anthocyanins produce their effects are essentially unknown. Thus, the aims of the present study were to test the hypothesis that sweet cherry anthocyanin (SCA) (a) reduces the release of inflammatory mediators from lipopolysaccharide (LPS)-stimulated RAW267.4 macrophages in vitro, and (b) prevent and/or reverse weight gain and inflammation in high fat fed C57BL/6J mice.

4.2 Materials and Methods

4.2.1 Cherry sourcing, selection and storage

Fresh *Prunus avium Lapin* cherries were sourced from Reid Fruits (Plenty, Tasmania, AUS). The cherries were washed and de-stemmed by hand, then de-pitted using a Steinomat hand processer (Westmark, Lennestadr-Elspe, GER) before being individually snap frozen in liquid nitrogen. The cherries were then stored at -80^{o}C until required for processing and extraction of the anthocyanins.

4.2.2 Extraction of sweet cherry anthocyanins

The edible portion of sweet cherry fruit (100g) was homogenized in acidified ethanol (1L 0.1% 12 N HCl ethanol) and incubated for 90 minutes at 37^{o}C. The sample was vacuum filtered, and the solvent evaporated to 25 mL using a Buchi rotary evaporator (Merck, Sydney, AUS) at 39^{o}C before being filtered through 45 µm then a 20 µm syringe filter. All samples were stored at -80^{o}C for a maximum of 6 months before being assayed by ultra-performance liquid chromatography (UPLC) as previously described (186) and included in chapters 2 and 3 of this thesis. As per section 3.2.4 of this thesis, characterisation and quantitation via UPLC was undertaken on every sample prior to usage in *in vitro* and *in vivo* experiments to ensure reliability of anthocyanin extract. The relative proportions of the identified anthocyanins (cyanidin-3-glucoside; cyanidin-3-rutinoside; pelargonidin-3-rutinoside; peonidin-3-rutinoside) which made up 90% of the total in the SCA extract, remained constant throughout, regardless of whether the extract was for treating mice or cells (see appendix 6). Whilst the overall TAC differed across extractions these were adjusted to ensure doses were kept constant.

4.2.3 In vitro studies to determine efficacy of sweet cherry anthocyanin in reducing inflammation

4.2.3.1 Cells

RAW264.7 murine macrophages were donated by Associate Professor Adele Holloway (School of Medicine, University of Tasmania, Hobart, AUS). Cells were cultured in Falcon Tissue Culture Flasks (Corning, Victoria, AUS) in Rosewell Park Memorial Institute (RPMI) media supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS; Fisher Biotec, Perth, AUS) and 2% penicillin-streptomycin stabilised solution (Sigma Aldrich, St Louis, USA) in a 5% CO_2 incubator at 37^oC. Cells were seeded at approximately 1 x 10^6 cells per 75cm^2 flask and were subcultured when at 80% confluence (passaged a maximum of 10 times) by cell-scraping then re-plating at ¼ cell density.

4.2.3.2 Cell viability assay to determine SCA concentration for use in vitro investigations

Cells were pre-treated with SCA (0 – 500 μM) for 24 hours at 37^oC, with H_2O_2 acting as a blank in the assay. Cell viability was assessed using the MTT Cell Proliferation Assay (Cayman Chemical Company, Ann Arbor, USA) as per manufacturer's instructions. Results indicated that up to 100 μM SCA did not adversely affect the growth of RAW264.7 macrophages, and as such this concentration was used for in vitro investigations (appendix 7).

4.2.3.3 Analysis of inflammatory mediators

RAW264.7 cells were incubated with SCA (100 μM) for 1 hour, followed by stimulation with LPS (1μg/mL; Sigma Aldrich, St Louis, USA) for 24 hours (28). After 24 hours, cells were harvested via scraping and the suspension centrifuged. Both the

supernatant and cell pellet were collected and stored at -80^{o}C until ELISA analysis. Concentrations of the inflammatory mediator's interleukin 6 (IL-6), interleukin 10 (IL-10) and granulocyte macrophage colony stimulating factor (GM-CSF) in the culture media were measured using ELISA (Jomar Life Research, Carribean Park, AUS) according to manufacturer's instructions. All samples were assayed twice in triplicate and with a blank to ensure assay reliability. Data were expressed as the mean $\pm$ SEM.

4.2.4 In vivo studies

4.2.4.1 Animals

All experimental procedures were approved by the University of Tasmania Animal Ethics Committee (A0015582) and performed in accordance with the Australian code of Practice for the Care and Use of Animals for Scientific Purposes – 2013, 8th Edition (218).

Male C57BL/6J adult mice (n=96) were obtained from the University of Tasmania Central Animal Facility (Cambridge, Tasmania, AUS). Ear-marked mice were housed in groups of between three to five utilising Opti mouse caging in accordance with Tasmanian Animal Ethics requirement to house mice in groups. Mice were kept in a 12 hr-12 hr light-dark cycle with ad libitum access to food and water in the Medical Science Precinct animal house (Hobart, Tasmania, AUS). On arrival, mice were acclimatised to the animal facility for at least one week before starting experimental diets, during which time they were provided water and standard chow ad libitum and monitored daily for adverse clinical signs (distress/ ill health) as per animal ethics requirements.

4.2.5 Study design

In order to test the effects of SCA on obesity and inflammation, following acclimatisation, mice were randomly allocated to one of two experimental arms - 'Prevention' (PT) or 'Reversal' (RT) and further allocated to treatment (Tx) or control group (Cx) within each arm of the trial (Fig 4.1). The six-week prevention (PT n=48) and ten-week reversal (RT n=48) trials were conducted in parallel.

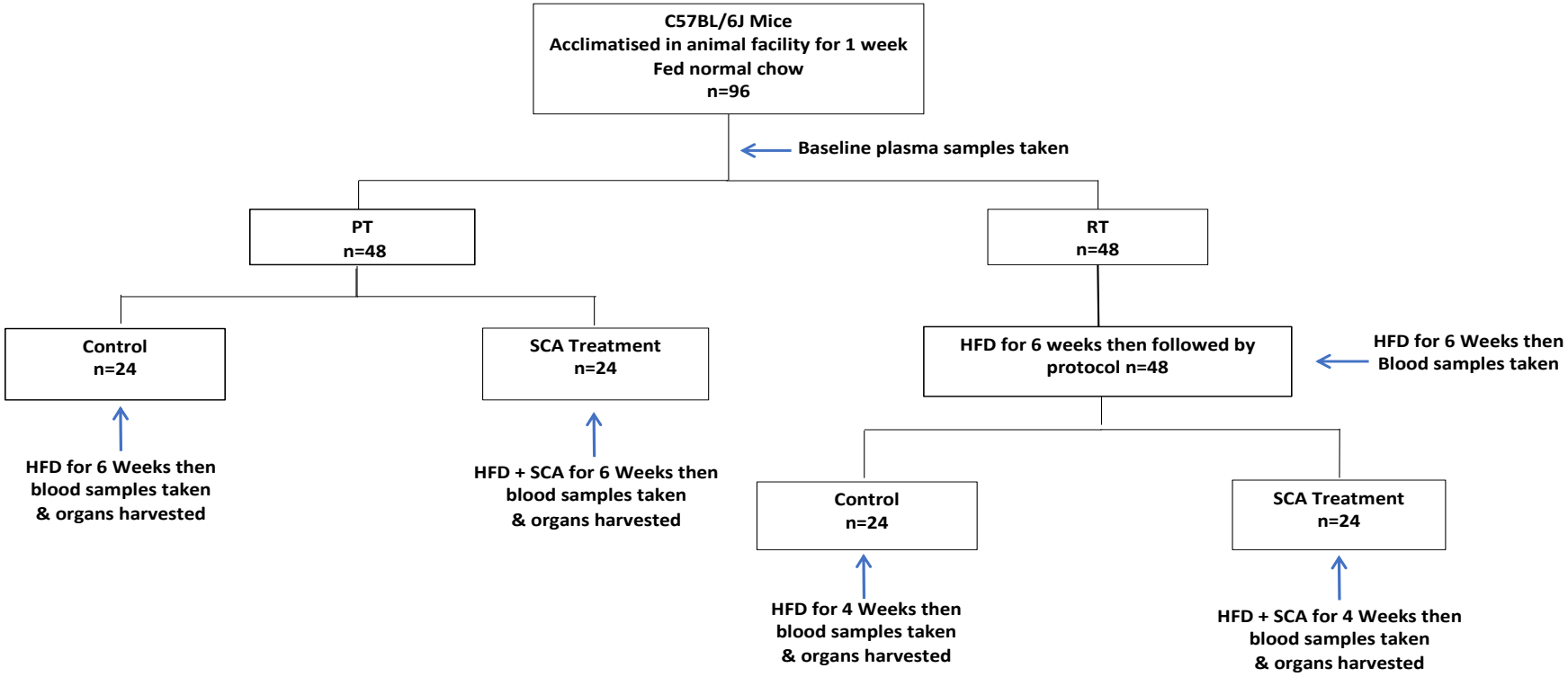


Figure 4.1 Study protocol for in vivo studies.

* All mice were fed a HFD for the duration of the study in both PT (6 weeks) and RT (10 weeks). In the PT, mice in the treatment arm (Tx) had SCA added to their drinking water at the start of week one (day 1) and it was maintained for 6 weeks. In the RT, all mice were fed a HFD and at the end of 6 weeks mice were divided into Control (Cx) and Tx groups. Tx mice had SCA added to their water at the end of week 6 (day 43) and this was continued for 4 weeks.

4.2.6 Diet composition

Each group received commercially prepared high fat rodent chow (SF03-002; kcal: fat 59%, carbohydrate 26%, protein 15%; Specialty Feeds, Glen Forrest, AUS) and water ad libitum. SCA at a dose of 40mg per kg body weight per day (64), was added to the water for the treatment phase of both prevention and reversal trials.

4.2.7 Measurements

Body weight, food and water intake were measured weekly. Fresh chow was provided to the mice twice per week. Body weight calculations were made on groups(s) of 24 or similar. Based on experimental design, food intake and water intake were calculated on groups of 6 (n = 6) as there were 3-4 animals per cage and so total food and water per cage divided by number of animals per cage). Blood was collected using EDTA-coated tubes by submandibular bleed (at baseline, midpoint and end of the trial). Blood glucose was measured using an Accu-check handheld monitor (Roche; CHE). At the conclusion of the study (6 weeks for PT or 10 weeks for RT), mice were exsanguinated via cervical dislocation. Epididymal fat pads (EFP) were removed, weighed and stored at -80^{o}C until analysis as an additional measure of obesity. Blood samples were centrifuged at 1000g for 15 min at 4^{o}C to separate plasma. Plasma was transferred to a clean tube and re-centrifuged at 10,000 g for 10 min at 4^{o}C, followed by separation into 30μL aliquots and stored at -80^{o}C until analysis. Plasma concentrations of IL-1β, IL-6, IL-10, TNFα, GM-CSF and MIP-2 were analysed at Cardinal Bioresearch (Brisbane, Queensland, AUS) via multiplex assay (Milliplex; Merck, Burlington, USA).

4.2.8 Data analysis

Values are presented as mean ± SEM and statistical analysis was performed using SPSS (IBM, USA). Outliers were identified as mice with results more than two SD from the mean in multiple variables and were omitted from analyses. Comparisons between groups in vivo trial were made using One-way ANOVA, followed by Student two-tailed t-test on identification of a significant ($p<0.05$) ANOVA.

4.3 Results

4.3.1 Effect of SCA treatment on inflammatory mediators in RAW264.7 macrophages in vitro

Stimulation of RAW264.7 macrophages with LPS increased the secretion of inflammatory mediators. Following treatment with SCA, LPS stimulated IL-10 and GM-CSF release was significantly reduced (Fig 4.2). The IL-10 concentration in the culture media of treated cells was 69.6% lower than that of untreated cells ($p = 0.02$; Fig 4.2a), whereas the GM-CSF concentration was 46% lower in treated cells ($p<0.001$; Fig 4.2b). No significant difference in LPS-induced secretion of IL-6 was observed between treated and untreated cells (Fig 4.2c).

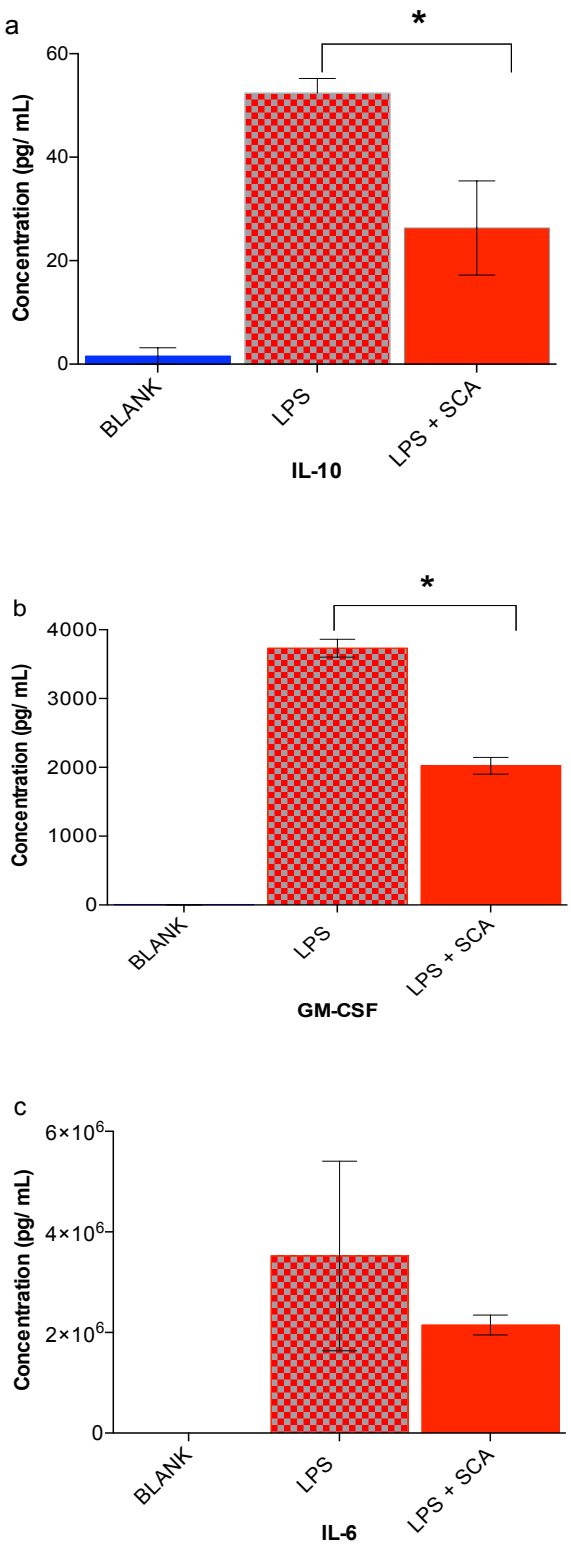


Figure 4.2 (a, b, c) Release of inflammatory mediators was measured in LPS stimulated RAW264.7 macrophages, with comparison made between sweet cherry anthocyanin (SCA) treated and untreated cells. Results are expressed as mean ± SEM (*p = 0.02; Fig 4.2a, *p<0.001; Fig 4.2b).

4.3.2 In vivo studies to determine the effect of sweet cherry anthocyanin on weight gain and inflammation

On completion of both arms of the mouse trial, no adverse clinical signs from either the high-fat diet or SCA were observed. Baseline characteristics of body weight, water and food intake were similar in the mice allocated to each trial (Table 4.1). Three mice out of 96 were excluded from analyses – one died from causes not attributable to the study and two were identified as outliers (due to growth being more than 2 SD from the mean).

Table 4.1 Observational data of body weight, food intake and water intake at baseline for prevention and reversal trials

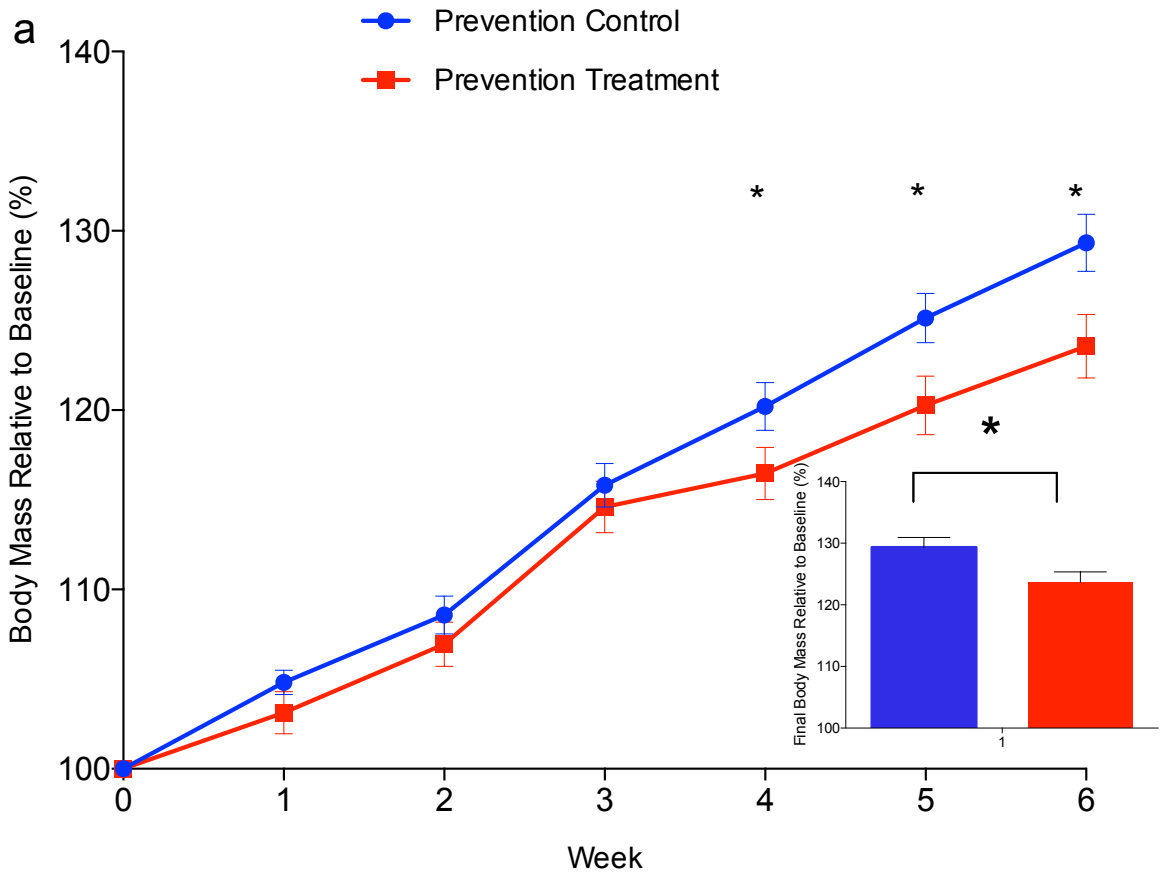
Variable	Prevention Trial (PT)			Reversal Trial (RT)		
	Control (Cx)	Treatment (Tx)	p-value[a]	Control (Cx)	Treatment (Tx)	p-value[a]
n	23	24		24	24	
Body weight (g)	28.0 (0.46)	28.2 (0.34)	0.80	28.1 (0.38)	28.6 (0.42)	0.41
n*	6	6		6	6	
Food intake[b]	1.60 (0.03)	1.63 (0.08)	0.80	1.53 (0.12)	1.73 (0.25)	0.52
Water intake[b]	0.90 (0.02)	0.90 (0.04)	0.84	1.03 (0.08)	0.92 (0.04)	0.26

a Constitutes the p-value of Cx versus Tx in both PT and RT

b Constitutes grams per gram body weight per week. * Food intake and water intake were calculated on groups of 6 (n = 6) as there were 3-4 animals per cage and so total food and water per cage divided by number of animals per cage).

4.3.3 Effect of sweet cherry anthocyanin on body weight in C57Bl/6J mice

No significant difference in weight gain was observed between the treatment and control groups during the first 3 weeks of the PT (Fig 4.3a). Between weeks 4 – 6, weight gain in the treatment group was significantly lower than that of the control group. Following 6 weeks of SCA consumption, PT-Tx mice had gained 19% less weight than PT-Cx mice ($p = 0.02$). No significant differences in weight gain were observed between treatment and control groups in the RT ($p > 0.05$; Fig 4.3b).



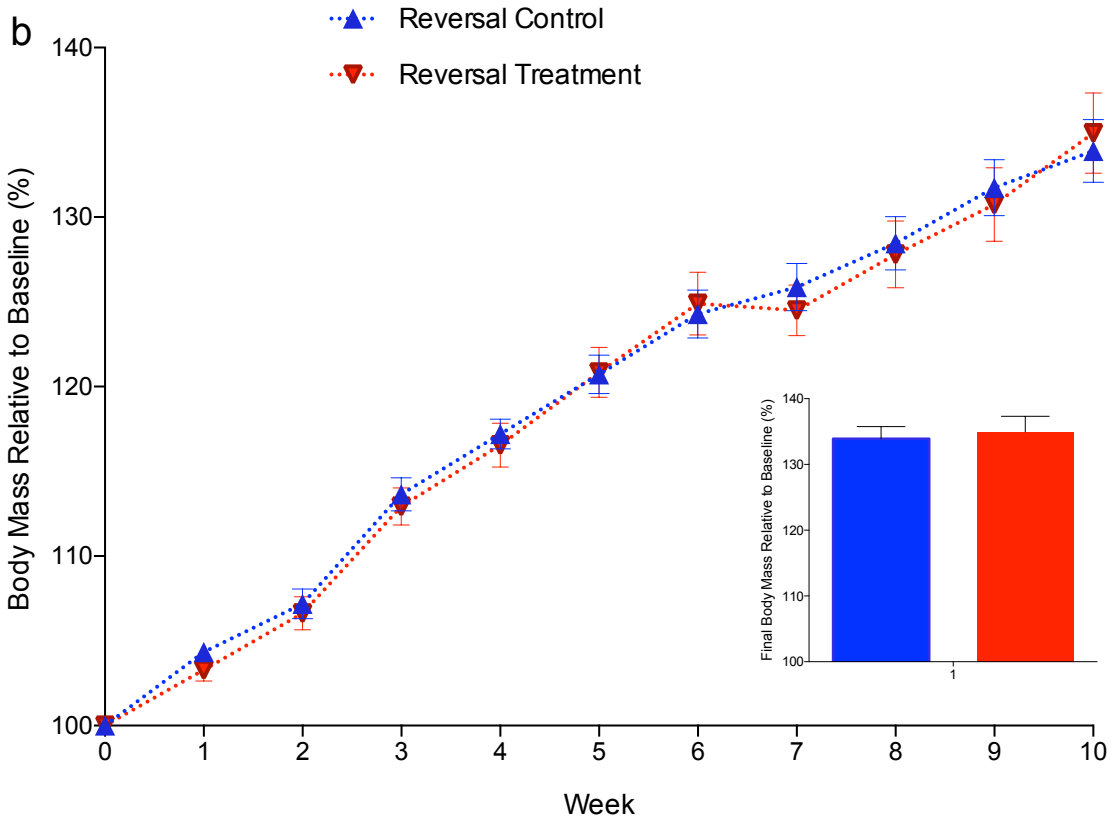


Figure 4.3 (a & b) Body weight of the mice was measured and compared between control and treatment groups in (a) prevention trial (PT) and (b) reversal trial (RT).

Results were expressed as mean ± SEM (*p = 0.02).

4.3.4 Effect of consumption of sweet cherry anthocyanin on food and water intake

In the PT there was no significant difference observed in food consumption between PT-Cx and PT-Tx groups ($p > 0.05$; Fig 4.4a). However, increased water consumption was observed in the PT-Tx group ($p = 0.01$; Fig 4.4b). Similar results were found in the RT, with no difference in food consumption (Fig 4.4c). Noteworthy, there was an increase in water consumption in the RT (Fig 4.4d), which was observed at the start of week 7 (the commencement of the treatment phase).

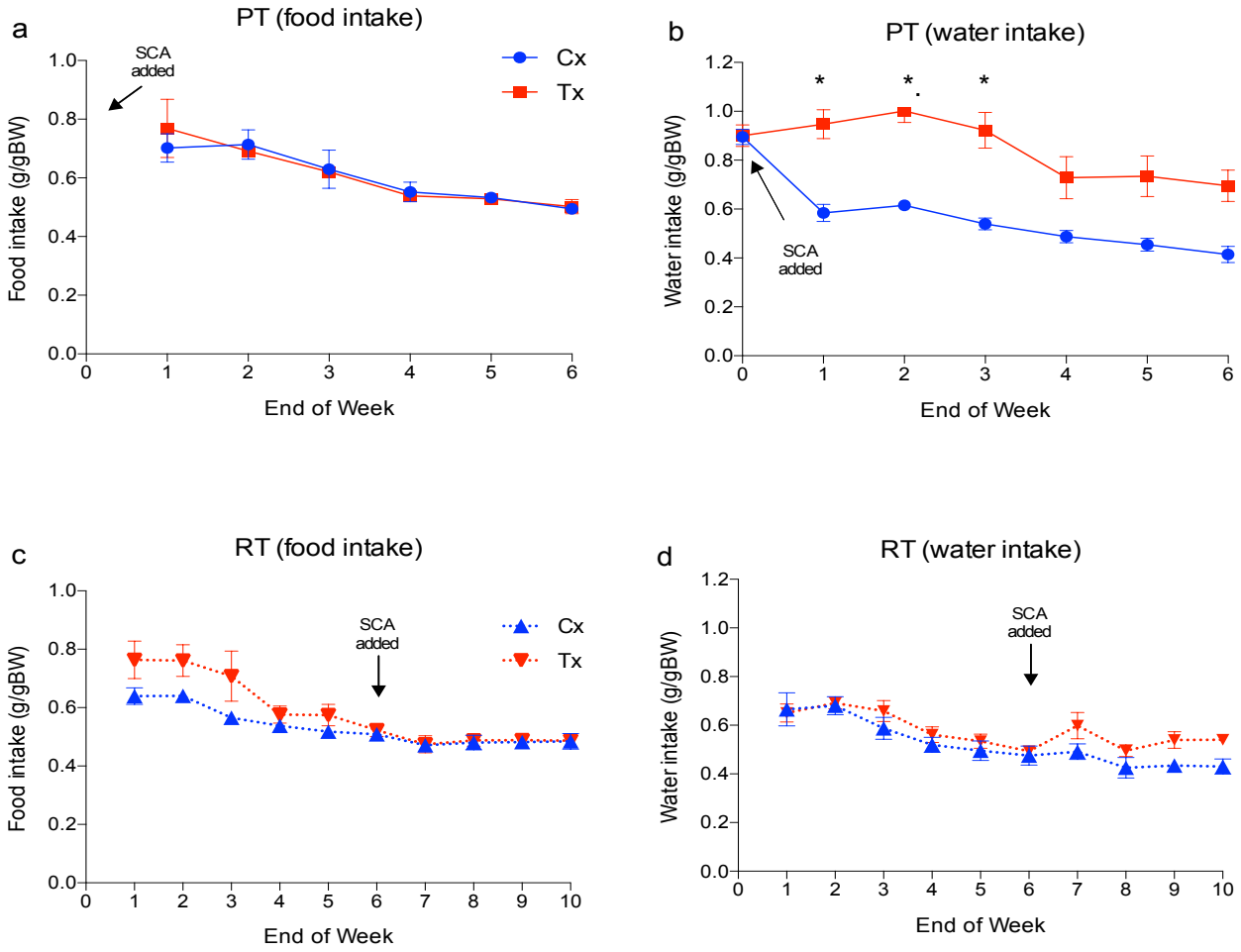


Figure 4.4 Food and water consumption were observed in prevention arm of the trial (a and b) and the reversal arm of the trial (c and d).

Water consumption (4b) was significantly increased in PT when comparing Cx to Tx (*p =0.01). Results are expressed as mean ± SEM.

4.3.5 Effect of consumption of sweet cherry anthocyanin on epididymal fat pad (EFP)

No significant difference in EFP absolute weight was observed between PT-Cx (1.87 ± 0.12g) and PT-Tx (1.64 ± 0.10g, p=0.14). A similar result was observed in the RT, with RT-Cx (1.94 ± 0.10g) and RT-Tx (2.13 ± 0.12g; p = 0.22).

4.3.6 Effect of consumption of sweet cherry anthocyanin on inflammatory markers in the prevention trial

The following panel of inflammatory markers were analysed to investigate the effect of SCA on HFD-induced inflammation in C57BL/6J mice in the PT (Fig 4.5): IL-6, IL-10, IL-1β, MIP-2, GMCSF and TNFα. Consumption of a HFD for 6 weeks resulted in significant increases in plasma IL-6 in both the PT-Tx (a 4-fold increase, $p = 0.03$) and PT-Cx groups (a 23-fold increase, $p < 0.001$) relative to baseline. Treatment with SCA significantly dampened the increase in IL-6 as compared with PT-Cx at the 6-week time-point ($p = 0.02$; Fig 4.5a). A similar trend was observed in plasma IL-10 concentrations at 6 weeks, however this difference was not statistically significant ($p = 0.08$; Fig 4.5d). No significant difference was observed in IL-10, IL-1β, TNFα or MIP-2 (Fig 4.5b-f).

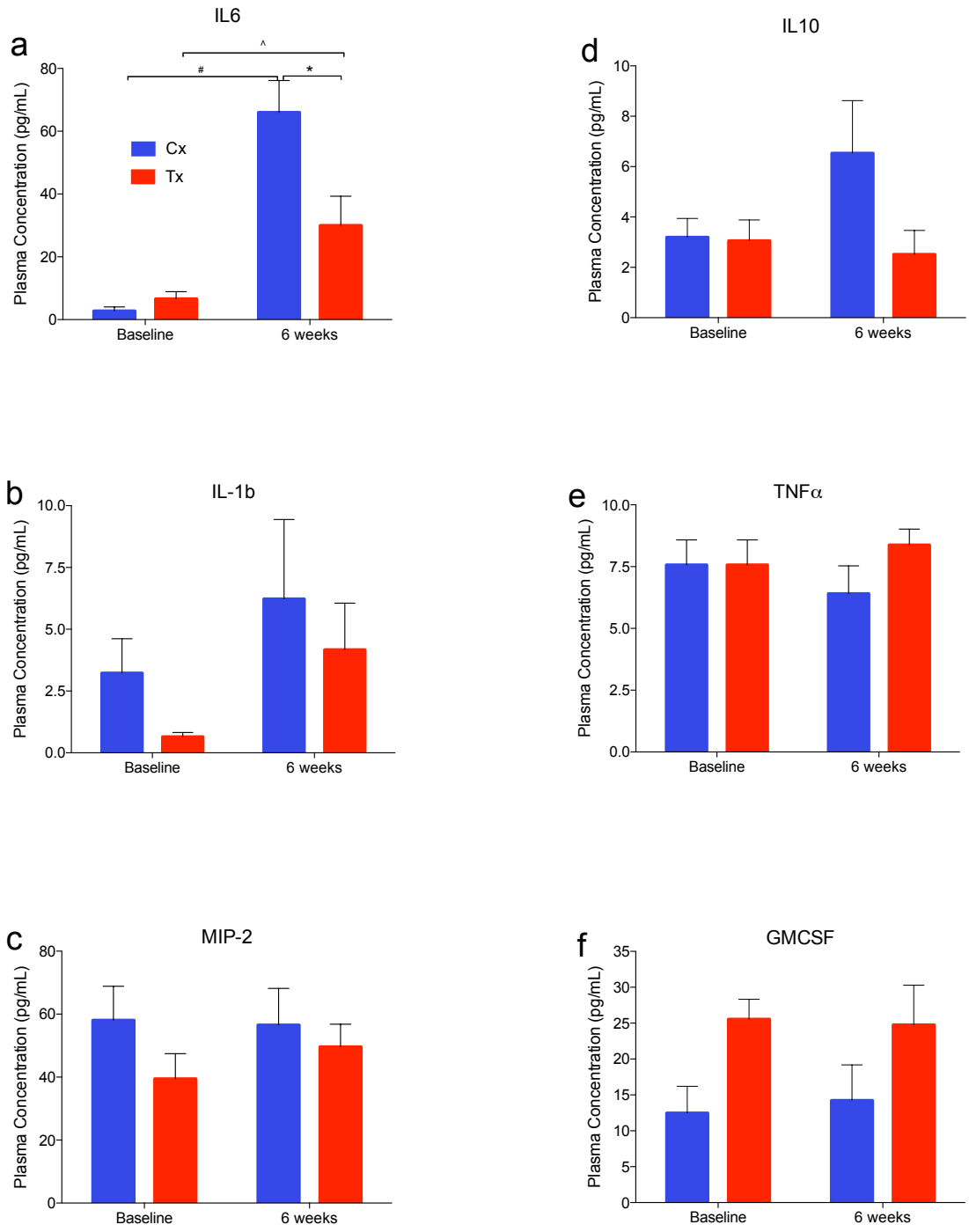


Figure 4.5 Comparison of inflammatory markers observed between control (Cx) and treatment (Tx) in the prevention trial (PT). Results are expressed as mean ± SEM (^p = 0.03, #p < 0.001, * p = 0.02).

4.3.7 Effect of consumption of sweet cherry anthocyanin on inflammatory markers in the reversal trial

In the reversal trial, treatment with SCA for four weeks resulted in a significant decrease from baseline in GM-CSF plasma concentration ($p = 0.02$; Fig 4.6a). On the contrary, an upward trend in GM-CSF concentration was observed in RT- Cx mice, resulting in a significant difference between the two groups at 10 weeks ($p < 0.001$). Similarly, the IL-10 concentration was significantly lower in the RT-Tx group as compared to the RT-Cx group at 10 weeks ($p = 0.01$; Fig 4.6d). No significant differences in IL-6, IL-1β, TNFα or MIP-2 were observed between the control and treatment groups at the 10-week time point (Fig 4.6b, c, e, f).

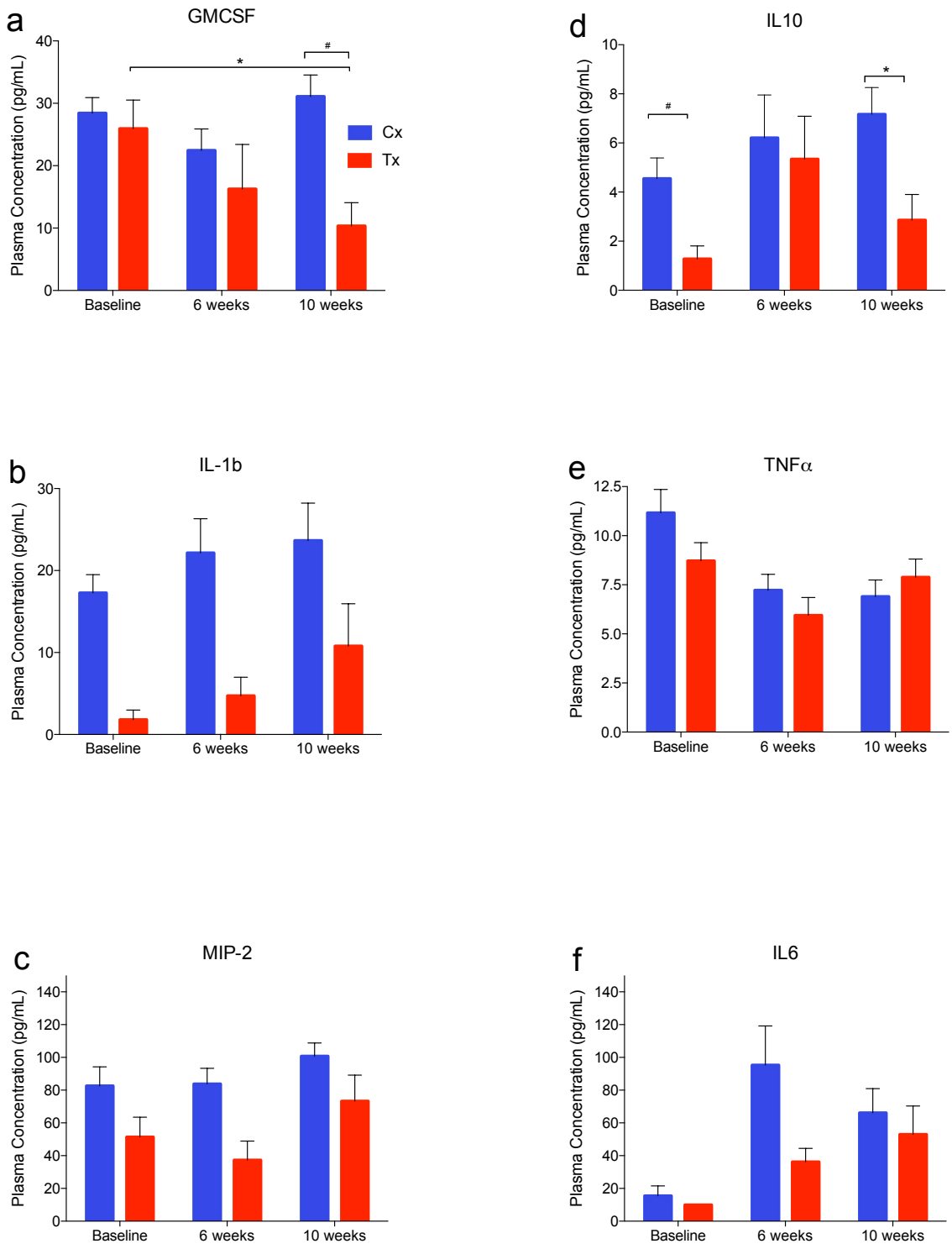


Figure 4.6 Comparison of inflammatory markers observed between control (Cx) and Treatment (Tx) in the reversal trial (RT). Results are expressed as mean ± SEM (*p = 0.02; Fig 4.6a, *p = 0.01; Fig 4.6d).

4.4 Discussion

This study yielded three equally important results: SCA significantly reduced the secretion of IL-10 and GM-CSF in vitro from RAW 264.7 cells, SCA significantly reduced weight gain in high fat fed mice and SCA attenuated the HFD-induced inflammatory response. The potential of SCA to provide a non-pharmacological solution to the burden of obesity and inflammation has global importance.

The effect of SCA on inflammatory marker release was investigated initially in vitro in RAW 264.7 macrophages. Reduction of pro-inflammatory markers with SCA has also been observed in a previous study by Alvarez-Suarez et al. (28) who found 40 – 50 μg/ml dose of cherry extract reduced secretion of inflammatory markers by approximately 50% in RAW 264.7 macrophages compared to controls. However, the cherry extract utilised in their work was from the Capulin cherry cultivar (*Prunus salicifolia*), which is a hybrid cherry/ plum and not a sweet cherry, which would be testing effectiveness of fruit with a different bioactive profile.

Reduction in inflammatory markers and systemic reduction of inflammation was observed in mice treated with SCA, which could have been attributed to either reduced secretion from adipocytes or reduced secretion from macrophages, or both. By undertaking the in vitro work, we posit that macrophages and other inflammatory cells are contributing to this reduced secretion, at least in part. The reduction in GM-CSF and IL-6 in the RT indicates that SCA reduces systemic inflammation, although this was observed without a concurrent reduction in adiposity as indicated through no significant difference in EFP between Cx and Tx groups. GM-CSF has previously been suggested to act as both a recruiter and activator of macrophages in adipose tissue, due to GM-CSF being directly correlated with the number of macrophages in visceral

fat tissues, as well as levels of circulating pro-inflammatory cytokines (28, 219). Kim et al. (219) investigated this further in a GM-CSF knockout mouse model with results suggesting a decreased level of circulating GM-CSF was able to reduce HFD-induced inflammation, which is in agreement with the current study. These significant findings from in vitro studies were unique in that sweet cherries were the source of anthocyanin, and they also provided the basis for exploring further the impact of SCA in vivo.

C57BL/6J mice fed SCA with HFD for 6 weeks as part of the prevention regime showed reduced weight gain as compared to HFD controls. This reduction in weight gain was not associated with a reduction in food intake. These results are consistent with previously published research, which has found reduced weight gain without a decrease in food consumption following treatment with anthocyanin extracts from varying sources including *Prunus avium* L (sweet cherry) and *Carpobrotus rossii* (27, 220, 221). When considering causality, Wei et al. found that Cy-3-G-treated mice had elevated plasma and skeletal lipoprotein lipase (LPL), but lower LPL in visceral adipose tissue. They suggested that the reduced adipose to skeletal muscle LPL ratio directs circulating lipids to skeletal muscle, and hence lowers fat accumulation in the adipose tissue (220).

Whilst the reduction in weight gain in the prevention group was not associated with significantly reduced food consumption, there was a significant increase in water consumption in the SCA group, which is an expected finding for this trial based on work by other researchers (68). Prior and colleagues undertook an anthocyanin supplementation trial using black raspberry as their source and also reported trends of attenuated weight gain and increased water consumption (68) in animals consuming anthocyanins. The sole purpose of Prior's experiments was to distinguish

between different anthocyanins from purified source and a “crude” black raspberry juice derivative in high and low-fat diets to determine change on body weight (68). Of interest in their work was that liquid consumption decreased in the high-fat fed mice (in both juice and purified anthocyanin treatments) compared with low-fat controls and in low-fat treated mice the water consumption was greater in juice treated group compared with purified anthocyanin treated group. This indicates a similar finding to our work of the high palatability (in the absence of adverse effects) of juice-based extract and whilst different diet composition was outside the scope of this body of work, it would be advantageous to identify whether the “crude” SCA extract utilised in these experiments created a similar effect in different dietary regimes.

Whilst the fluid intake increased, the relationship between fluid and food consumption is worthy of further scrutiny. One possible explanation is that “crude” SCA extract has a diuretic effect, which leads to a reactive increase in fluid consumption, albeit insufficient to compensate for the fluid loss. The nature in which animals were housed for this study did not allow accurate estimation of urinary output. However, in hindsight this would have been a useful measure as it would have confirmed the postulated diuretic effect of SCA. This diuretic effect has been shown in research on *Hibiscus sabdariffa*, which also has high anthocyanin content (222). Furthermore, a double blind study by Hooman and colleagues (223) resulted in significant diuretic effect after 24 hour administration of capsules containing 2g sour cherry stalk. While the mechanism is not known, this finding is significant both in a scientific sense and also in the context of future research where these findings would be translated within a clinical context.

In both treatment and control groups, a number of inflammatory markers were increased at 6 weeks, which indicates that the high fat diet induced inflammation.

However, the inflammatory response in the PT-Tx mice was significantly dampened (compared to controls) at 6 weeks, which shows that SCA treatment was effective in reducing inflammation. Similarly, in the RT, SCA also caused a significant improvement in inflammatory state as shown by increased plasma GM-CSF and decreased plasma IL-10.

Observations from both the in vitro and in vivo studies suggest that the SCA are inhibiting production of GM-CSF from the macrophages at a cellular level. However, whether this is an intracellular or extracellular mechanism remains to be elucidated. An important facet of this research was exploring and investigating obesity and the associated inflammation through both in vitro and in vivo models. Similar research by Li and colleagues (34) explored the effect of an anthocyanin rich extract from red raspberries in a model of colitis in mice, showing that both in vitro to in vivo studies are warranted.

The novelty of this work exists in that the in vitro and in vivo work occurred as part of the one study. In recent work by other researchers, the effect of anthocyanins on markers of health and disease has been investigated through in vitro and in vivo measures, yet these have occurred in isolation and also have not utilised the same anthocyanin source (28, 30, 31, 44, 47, 49, 212, 224, 225). However, in this trial, both aspects were observed in sequence and the in vitro work informed the subsequent direction for the in vivo trial. Undertaking the work in this way provides a novel approach through added consistency of methods, treatment protocols and anthocyanin extract source.

Whilst it was outside the scope of this trial, the dosing regimen for the treatment periods in both arms of the trial may not be appropriate for humans. Although it was

effective in exhibiting a response, an equivalent dose for a 70 kg human, would be 2.8 g anthocyanins. This would only be possible through consumption of an extract and not whole fruit, and investigation into the tolerance of such a dose would be required. Future investigation into the impacts of different doses of sweet cherry anthocyanins would be critical, in order to determine any potential dose response relationship.

Limitations

The first limitation that confounded results between effect in prevention and reversal modalities, was the length of time in the treatment phase of the reversal arm. One of the outcomes of the trial was the significant reduction in weight gain in treated mice, observed in weeks 4, 5 and 6 of the prevention trial. The reversal trial design only allowed for 4 weeks treatment; as such it may be possible that the treatment effect was not observed due to the shortened treatment phase, which was a trial design fault. In future studies it would be of benefit to undertake a trial with extended treatment phases to determine if the length of treatment provided any additional benefit. There were significant differences in the baseline and midpoint measurements between the groups for some of the cytokines which is not a noteworthy result of effect, however in future research an appropriate protocol could address this.

4.5 Conclusion

It is evident from this study that sweet cherry anthocyanins may play a role in slowing the rate of weight gain and the associated chronic low-grade inflammation induced by a high fat diet. Further work on the cellular mechanism of action is warranted. Whilst translating these results to the human population is not without its challenges, there is a useful paradigm to be explored further.

Chapter 5: General discussion, future directions and conclusions

5.1 General discussion

The research described in Chapters 2 – 4 constitutes a body of work that is broad in content covering analytical chemistry; plant, cell and murine biochemistry and physiology; through to economics and commercial application of waste fruit. Through the course of the work a number of novel findings were generated.

The optimised extraction findings were in contrast to existing literature, in that attempts by others to manipulate process parameters for solvent based extraction had not achieved such a high yield. Retrieving more anthocyanin from the fruit has a direct benefit for producers. Even though there are other extraction methods that can potentially achieve a comparable yield, a method which can readily be reproduced outside the laboratory, without the requirement of highly specialised technical equipment was an important consideration to this work. This enables an increased likelihood of commercial level extraction, translating to economic viability for the industry.

Whilst the weather patterns identified have previously been reported in plant physiology research as a means to ensure tree growth and fruit yield, the applied context for these findings in relation to anthocyanin content, has not been considered until this current work. These studies enabled a clear picture of weather conditions that would likely result in greater amounts of anthocyanin and also weather patterns that would influence whether fruit is considered premium, waste or second grade fruit. Understanding the influence of weather patterns on the growth and maturation of the

fruit will potentially enable farmers to make earlier decisions about harvest priorities. If it is a season with less than ideal growing conditions and it is clear that the fruit is going to be soft and be deemed waste fruit, rather than pick the fruit conventionally by hand, which is labour intensive in order to protect the fragile nature of the fruit, other means of picking the fruit more efficiently could be explored, and could include mechanical harvesting. This information is vital when planning for season outcomes, allowing the necessary preparation to be undertaken by farmers so that waste fruit can efficiently be harnessed with less fruit contributing to landfill.

Another important and unexpected outcome of the work is the identification of Prunus avium L. 'Kordia' cultivar having significantly higher anthocyanin levels (up to 4-fold) than other cultivars. Whilst others have reported variation between cultivars, this has not been extensively studied in sweet cherries until now. This is relevant when decisions are made as to which cultivar is to be utilized for extraction and/or for its functional properties. This understanding of fruit with higher anthocyanin yield could also assist farmers when deciding which cultivar to propagate. Particularly when considering a cultivar which is more challenging to grow, like Prunus avium L. 'Kordia', the knowledge that the yield of anthocyanin is significantly higher could influence production decisions. Combine these factors with the finding of storage temperature and precipitous decline of anthocyanins as soon as 3 months after storage, a clear picture forms about how to better harness anthocyanins. It is now evident that storing the fruit for more than 12 months at -20^{o}C results in significant loss of anthocyanin. So, when faced with storing fruit before it is processed/extracted, it may be beneficial to consider waste fruit and of Prunus avium L. 'Kordia' cultivar for this, as it would enable a higher yield of recovery. Together, it provides strong rationale for the increased investment in a fruit that is difficult to grow as the economic benefit is

significant.

During the course of this research it was uncovered that a significant percentage of fruit does not make it to market, and ultimately ends up as waste, as landfill/rotting on the ground. This is a consequence of the fruit being deemed not fit for market, due to softness, splits and cracks. In each season this is estimated to be between 20-50% of the crop which is a significant volume that is currently of no financial value. In the present study, the waste fruit had significantly more anthocyanin that the premium grade fruit which is an important and novel finding for the sweet cherry industry. It transforms a waste product into an economically viable commodity that can be harnessed for financial benefit. This supports the consensus in other fruit species that waste fruit has bioactive components that render it useful for wider application.

Hundreds/thousands of tonnes of cherry fruit go to waste each year around the globe and this fruit now has the potential to be re-purposed through an effective extraction protocol guaranteeing high yield of anthocyanins. The re-purposing of waste will also ensure consumers have access to more anthocyanin year-round. Outside the consumption season (Dec – Feb) in Tasmania, they could access anthocyanins through fortification of other foods or through nutraceutical products that have been created from the waste. This will contribute to the overall anthocyanin profile in the diet with potential to impact inflammatory disease.

The final and possibly most significant outcome was that the sweet cherry extract was effective in preventing inflammation and reducing the amount of weight gained when consuming a high fat diet. The sugar content of the extract was high as expected given the extract was fruit derived. We used Brix testing which is commonly used both in the field and in the laboratory to quantify sugar levels. The sugar level of extract

was found to be 26 °Bx on the Brix scale which means there is a high level of sugar in the extract and effectively 26g sucrose per 100g solution (or 26% sugar). This would potentially mean that mice who were consuming the SCA extract had additional calories in the form of the extract added to their water. As we didn't account for this in the research design it is difficult to calculate exactly how many extra calories they consumed and should be considered for future experiments. However, it does provide a promising discovery as the mice treated with SCA had higher water consumption yet still consumed the same amount of food and gained less weight than matched controls. This suggests that treated mice consumed more calories from the sugar in SCA, yet they still gained 19% less weight than mice that did not consume SCA.

Whilst this is not a conclusive finding, given the nature of the evidence that surrounds sugar consumption and the impact this has on ill health and development of obesity, the high sugar concentration of the extract could be considered favourable oversight and warrants further investigation.

This is in support of previous work carried out both *in vitro* and *in vivo*, where disease processes such as obesity and inflammation can be reduced. Whilst the results were significant, the clinical application has greater ongoing potential, as the reduction in weight gain at 19% far superseded expectations and if extrapolated and translated into human research would potentially allow for good patient outcomes. With the global incidence of obesity and inflammation at an alarmingly high rate, and billions of people that could benefit worldwide, the potential to be able to reduce obesity using a waste product is certainly desirable.

5.2 Limitations

5.2.1 Intervention length & composition

The length of the mouse intervention/supplementation trials may be pivotal when considering the effect of SCA on obesity and inflammation. Whilst significant findings were observed in the prevention trial, we found that these occurred between 4 - 6 weeks. By the nature of the research design and based on prior research, 4 weeks was hypothetically long enough to see an effect. However, as the trials were run in parallel (not in sequence), the crucial 4 – 6 week observation was not identified until post trial. Whilst a positive trend was observed (unpublished), if the reversal trial arm had been extended for an additional 2 weeks this may have allowed for significant outcomes in SCA ability to reverse the damage of a high fat diet. Furthermore, s

5.2.2 Weather measurements

The retrospective analysis of weather patterns and their influence on TAC uncovered crucial season specific weather events and conditions that warrant further investigation. This was not a pre-planned part of this investigation as environmental/weather conditions were not considered relevant until it was found that variation in anthocyanin does occur across different growing seasons. Upon reflection more precise measurements of rainfall and temperature at the site of production would have been ideal. Rather than retrospectively extrapolated data from nearby weather stations, direct measurement of rainfall and temperature would strengthen the argument for causation by seasonal changes and improve the veracity of the work.

5.2.3 Waste fruit usage

Due to time constrictions, the studies were designed in parallel rather than in

sequence. Had the studies had been designed and undertaken in sequence, the waste fruit outcome would have been identified and then all subsequent research undertaken in waste fruit (as opposed to premium fruit). Ideally, waste fruit would have been utilised across all aspects of the study. This would enable a stronger more definitive message for waste fruit usage.

5.2.4 Extract constituents

Whilst it appears the findings are a result of bioactive compounds, anthocyanins being one of those, as we did not undertake and examine the individual components in the SCA extract which provides a challenge to understanding the true nature of the extract utilised. We recognise there are other components (not just anthocyanins) potentially creating this effect. With future experimental design, deeper scrutiny of the extract including other molecules present, the level and differences in sugar concentration across cultivars and extracts, plus the synergistic impact of those factors is warranted and is lacking from the current research design. This could be accounted for with experiments developed comparing crude extract with a purified sample with additional steps to isolate and quantify other bioactive compounds that may be present.

5.3 Outside the scope of this body of work

5.3.1 Geographical variation

As a control measure to improve research rigour, all studies were based on fruit from one geographical location. For future studies, comparison of fruit that are grown in a range of geographical locations, and presumably different ‘micro-climates’ would test and inform any variation in SCA levels based on growing region. It is evident from the literature, that weather conditions are particular to geographical region (81) and that

the differences in weather patterns can impact fruit growth. This research did not investigate or compare effect of micro-climates on growth and SCA. This has the potential to impact yield and waste to market ratio. The current study design did not allow for independent measurement and analysis of the environment including specific micro-climates, soil and plant physiology measures. The nature of this trial also had cherry samples provided from one geographical location. This was advantageous as the growing conditions (other than weather) were constant and this provided strong intra-sample reliability. However, with a lack of geographical diversity in the sampling, it could mean the translation of results to other areas difficult.

5.4 Future studies

5.4.1 Longer intervention with dose and extract variation

Improvement in obesity and inflammatory status with high dose SCA warrants scrutiny at lower doses. It would be beneficial to understand the effect of different doses through in vitro and in vivo trials. This would enable clarity around minimum dose required to create an effect, and if designed with whole fruit and extract in a parallel design, would provide real transferability to the general consumer. As part of a longer intervention, toxicity studies would be required and part of study design to ensure no negative effect from long-term consumption. The strength of this body of work was in the multilayered methodology applied to understand aspects such as cultivar variation, growing conditions and extraction optimisation. Whilst the research design for this body of work did not test a range of different diets and conditions, to be able to determine whether SCA does create a diuretic effect, and also to better understand the impact of calories, future work would benefit from a more complex supplementation protocol. In line with other research in the space, it would be pertinent to consider

research design involving different dietary patterns including calories and a purified anthocyanin extract (with sugars removed) OR conversely if suing a crude extract to supplement the diet with additional sugar in the control mice to be able to determine the impact and mechanism of SCA.

5.4.2 Trials of whole fruit and human trials

The outcomes were based on the extraction of anthocyanin and administering that extract across supplementation trials. In order to expand the practical application of the work, investigation into effect of whole fruit consumption on markers of disease would be warranted. This could be undertaken in mice, with a natural progression to a clinical trial to explore the effectiveness of the sweet cherry anthocyanin extract on measures of disease in humans. Specifically, investigation into the effects of the extract on acute and chronic inflammation, gut microbiota and in diseases with an inflammatory basis such as gout, arthritis and obesity.

5.4.3 Investigation into alternate methods for capturing SCA

Whilst effective for this body of work, future study design to encompass direct capture of fruit waste to market ratio would be beneficial. If it is possible to determine areas with higher waste: market ratio, this would allow potential strategies to harness that waste with economic viability and efficiency. Specifically, it would also be beneficial to explore methods for preserving SCA in the fruit. Specifically, freeze-drying (for human consumption), juicing and alternate methods of extraction.

5.5 Concluding comments relative to outcomes

Novel findings from the research undertaken in this thesis have clearly shown there are direct health and economic benefits from utilising cherry waste. The optimised

extraction of the anthocyanins has commercial significance and the sweet cherry extract has potential as an adjunct therapy for obesity and inflammation. The potential for these findings to be translated into clinical practice to improve patient care is an exciting prospect. To date, the links between all the components studied in this thesis have not been made, particularly the exploration from paddock to plate (or pill) including extraction and functional testing in cells and animals. Add to this the outcomes of environmental sustainability and financial viability for the farmers for utilising waste fruit, and this work grows in importance. This research links and provides evidence as to sweet cherry having a significant functional role that with further investigation, could contribute to the addressing the global obesity epidemic. In conclusion, this body of work provides significant evidence that Tasmanian sweet cherries are effective functional foods and their use, with direct health and economic benefits, is warranted.

References

1. Caleja C, Ribeiro A, Barreiro MF, Ferreira I. Phenolic Compounds as Nutraceuticals or Functional Food Ingredients. Curr Pharm Des. 2017;23(19):2787-806.

2. Martirosyan D, Singh JA. A new definition of functinoal food by FFC: what makes a new definition unique? Funct food health dis. 2015;5(6):209-23.

3. Shahidi F. Nutraceuticals, functional foods and dietary supplements in health and disease. J Food Drug Anal. 2012;20(SUPPL 1):226-30.

4. Shipp J, Adel-Aal E. Food application and physiological effects of anthocyanins as functional food ingredients. Open Food Sci J 2010;4:7-22.

5. Mouradov A, Spangenberg G. Flavonoids: a metabolic network mediating plants adaptation to their real estate. Front Plant Sci. 2014;5:1 - 16.

6. Crozier A, Jaganath IB, Clifford MN. Dietary phenolics: chemistry, bioavailability and effects on health. Nat Prod Rep. 2009;26(8):1001-43.

7. Panche AN, Diwan AD, Chandra SR. Flavonoids: an overview. J Nutr Sci. 2016;5(e47):1-15.

8. Khoo HE, Azlan A, Tang ST, Lim SM. Anthocyanidins and anthocyanins: colored pigments as food, pharmaceutical ingredients, and the potential health benefits. Food Nutr Res. 2017;61(1):1-21.

9. Gonzalez-Gallego J, Garcia-Mediavilla MV, Sanchez-Campos S, Tunon MJ. Fruit polyphenols, immunity and inflammation. Br J Nutr. 2010;104 Suppl 3:S15-27.

10. Khoo GM, Clausen MR, Pedersen BH, Larsen E. Bioactivity and total phenolic content of 34 sour cherry cultivars. J Food Compos Anal. 2011;24(6):772-6

11. HIA. Australian horticulture statistics handbook 2015/16. Sydney, Australia: Horticulture Innovation Australia; 2017 24/05/2017.

12. Aslam H, Raheem M, Ramzan R. Utilization of mango waste material (peel, kernel) to enhance dietary fiber content and antioxidant properites of biscuit. J Glob Innov Agric Soc Sci 2014;2(2):76 - 81.

13. Lee J, Wrolstad RE. Extraction of anthocyanins and polyphenolics from blueberry processing waste. J Food Sci. 2004;69(7):564-73.

14. Leroy BLMM, Bommele L, Reheul D, Moens M, De Neve S. The application of vegetable, fruit and garden waste (VFG) compost in addition to cattle slurry in a silage maize monoculture: Effects on soil fauna and yield. Eur J Soil Biol. 2007;43(2):91-100.

15. Malla BA, Rastogi A, Sharma RK, Ishfaq A, Farooq AJ. Kinnow madarin (Citrus nobilis lour x Citrus deliciosa tenora) fruit waste silage as potential feed for small ruminants. Vet World. 2015;8(1):19-23.

16. Hasler C. Functional foods: benefits, concerns and challenges - a position paper from the american council on science and health. J Nutr. 2002;132(12):3772-81.

17. Bray GA. Medical treatment of obesity: the past, the present and the future. Best Pract Res Clin Gastroenterol. 2014;28(4):665-84.

18. Rucker D, Padwal R, Li SK, Curioni C, Lau DC. Long term pharmacotherapy for obesity and overweight: updated meta-analysis. BMJ. 2007;335(7631):1-10.

19. Baboota RK, Bishnoi M, Ambalam P, Kondepudi KK, Sarma SM, Boparai RK, et al. Functional food ingredients for the management of obesity and associated co-morbidities – A review. J Funct Foods. 2013;5(3):997-1012.

20. Brown L, Poudyal H, Panchal SK. Functional foods as potential therapeutic options for metabolic syndrome. Obes Rev. 2015;16(11):914-41.

21. Hirai S, Takahashi N, Goto T, Lin S, Uemura T, Yu R, et al. Functional food

targeting the regulation of obesity-induced inflammatory responses and pathologies. Mediators Inflamm. 2010;2010:367838.

22. Motohashi N, Gallagher R, Vanam A, Gollapudi R. Functional foods and their importance in geriatric nutrition. J Clin Nutr Metab. 2017;1(1):1-5.

23. Cencic A, Chingwaru W. The role of functional foods, nutraceuticals, and food supplements in intestinal health. Nutrients. 2010;2(6):611-25.

24. Laparra JM, Sanz Y. Interactions of gut microbiota with functional food components and nutraceuticals. Pharmacol Res. 2010;61(3):219-25.

25. Henry CJ. Functional foods. Eur J Clin Nutr. 2010;64(7):657-9.

26. Azzini E, Giacometti J, Russo GL. Antiobesity effects of anthocyanins in preclinical and clinical studies. Oxid Med Cell Longev. 2017;2017:2740364.

27. Wu T, Tang Q, Yu Z, Gao Z, Hu H, Chen W, et al. Inhibitory effects of sweet cherry anthocyanins on the obesity development in C57BL/6 mice. Int J Food Sci Nutr. 2014;65(3):351-9.

28. Alvarez-Suarez JM, Carrillo-Perdomo E, Aller A, Giampieri F, Gasparrini M, Gonzalez-Perez L, et al. Anti-inflammatory effect of Capuli cherry against LPS-induced cytotoxic damage in RAW 264.7 macrophages. Food Chem Toxicol. 2017;102:46-52

29. Bhaswant M, Shafie SR, Mathai ML, Mouatt P, Brown L. Anthocyanins in chokeberry and purple maize attenuate diet-induced metabolic syndrome in rats. Nutrition. 2017;41:24-31.

30. Paquette M, Medina Larque AS, Weisnagel SJ, Desjardins Y, Marois J, Pilon G, et al. Strawberry and cranberry polyphenols improve insulin sensitivity in insulin-resistant, non-diabetic adults: a parallel, double-blind, controlled and randomised clinical trial. Br J Nutr. 2017;117(4):519-31.

31. Yang JH, Choi MH, Yang SH, Cho SS, Park SJ, Shin HJ, et al. Potent anti-inflammatory and antiadipogenic properties of bamboo (sasa coreana nakai) leaves extract and its major constituent flavonoids. J Agric Food Chem. 2017;65(31):6665-73.

32. Edirisinghe I, Banaszewski K, Cappozzo J, Sandhya K, Ellis CL, Tadapaneni R, et al. Strawberry anthocyanin and its association with postprandial inflammation and insulin. Br J Nutr. 2011;106(06):913-22.

33. Guo H, Xia M, Zou T, Ling W, Zhong R, Zhang W. Cyanidin 3-glucoside attenuates obesity-associated insulin resistance and hepatic steatosis in high-fat diet-fed and db/db mice via the transcription factor FoxO1. J Nutr Biochem. 2012;23(4):349-60.

34. Li L, Wang L, Wu Z, Yao L, Wu Y, Huang L, et al. Anthocyanin-rich fractions from red raspberries attenuate inflammation in both RAW264.7 macrophages and a mouse model of colitis. Sci Rep. 2014;4:1-11.

35. Ellingsen I, Hjerkinn EM, Seljeflot I, Arnesen H, Tonstad S. Consumption of fruit and berries is inversely associated with carotid atherosclerosis in elderly men. Br J Nutr. 2008;99(3):674-81.

36. Kapoor D, Munjal A. Functional Foods: The New Secret of the Health Conscious Indian Women!! Global Bus Rev. 2017;18(3):750-65.

37. Vella MN, Stratton LM, Sheeshka J, Duncan AM. Functional food awareness and perceptions in relation to information sources in older adults. Nutr J. 2014;13:44.

38. Dagevos H. Consumers as four-faced creatures. Looking at food consumption from the perspective of contemporary consumers. Appetite. 2005;45(1):32-9.

39. Urala N, Lähteenmäki L. Reasons behind consumers' functional food choices. J Nutr Food Sci. 2003;33(4):148-58.

40. Dever M, MacDonald R, Cliff M, Lane W. Sensory evaluatino of sweet cherry cultivars. HortScience. 1996;31(1):150 - 3.

41. Thornsbury S, Martinez L. Capturing demand for functional foods: a case study from the tart cherry industry. Am J Agric Econ. 2011;94(2):583-90.

42. Flores G, Dastmalchi K, Paulino S, Whalen K, Dabo AJ, Reynertson KA, et al. Anthocyanins from Eugenia brasiliensis edible fruits as potential therapeutics for COPD treatment. Food Chem. 2012;134(3):1256-62.

43. Dragano NR, Marques A, Cintra DE, Solon C, Morari J, Leite-Legatti AV, et al. Freeze-dried jaboticaba peel powder improves insulin sensitivity in high-fat-fed mice. Br J Nutr. 2013;110(3):447-55.

44. Garcia-Diaz DF, Johnson MH, de Mejia EG. Anthocyanins from fermented berry beverages inhibit inflammation-related adiposity response in vitro. J Med Food. 2015;18(4):489-96.

45. Hassellund SS, Flaa A, Kjeldsen SE, Seljeflot I, Karlsen A, Erlund I, et al. Effects of anthocyanins on cardiovascular risk factors and inflammation in pre-hypertensive men: a double-blind randomized placebo-controlled crossover study. J Hum Hypertens. 2013;27(2):100-6.

46. Qin B, Anderson RA. An extract of chokeberry attenuates weight gain and modulates insulin, adipogenic and inflammatory signalling pathways in epididymal adipose tissue of rats fed a fructose-rich diet. Br J Nutr. 2012;108(4):581-7.

47. Esposito D, Damsud T, Wilson M, Grace MH, Strauch R, Li X, et al. Black currant anthocyanins attenuate weight gain and improve glucose metabolism in diet-induced obese mice with intact, but not disrupted, gut microbiome. J Agric Food Chem. 2015;63(27):6172-80.

48. Farrell N, Norris G, Lee SG, Chun OK, Blesso CN. Anthocyanin-rich black

elderberry extract improves markers of HDL function and reduces aortic cholesterol in hyperlipidemic mice. Food Funct. 2015;6(4):1278-87.

49. Hoek-van den Hil EF, van Schothorst EM, van der Stelt I, Swarts HJ, van Vliet M, Amolo T, et al. Direct comparison of metabolic health effects of the flavonoids quercetin, hesperetin, epicatechin, apigenin and anthocyanins in high-fat-diet-fed mice. Genes Nutr. 2015;10(4):1-13.

50. Matias AA, Rosado-Ramos R, Nunes SL, Figueira I, Serra AT, Bronze MR, et al. Protective effect of a (poly)phenol-rich extract derived from sweet cherries culls against oxidative cell damage. Molecules. 2016;21(4):1-16.

51. Lynn A, Mathew S, Moore CT, Russell J, Robinson E, Soumpasi V, et al. Effect of a tart cherry juice supplement on arterial stiffness and inflammation in healthy adults: a randomised controlled trial. Plant Foods Hum Nutr. 2014;69(2):122-7.

52. Song H, Wu T, Xu D, Chu Q, Lin D, Zheng X. Dietary sweet cherry anthocyanins attenuates diet-induced hepatic steatosis by improving hepatic lipid metabolism in mice. Nutrition. 2016;32(7-8):827-33.

53. Prior R, Wilkes E, Rogers R, Khanal R, Wu X, Howard L. Purified blueberry anthocyanins and blueberry juice alter development of obesity in mice fed an obesogenic high-fat diet. J Agric Food Chem. 2010;58(7):3970-6.

54. Kong JM, Chia LS, Goh NK, Chia TF, Brouillard R. Analysis and biological activities of anthocyanins. Phytochemistry. 2003;64(5):923-33.

55. Pervaiz T, Songtao J, Faghihi F, Haider MS, Fang J. Naturally occurring anthocyanin, structure, functions and biosynthetic pathway in fruit plants. Plant Physiol Biochem. 2017;05(02):1-9.

56. Visioli F, De La Lastra CA, Andres-Lacueva C, Aviram M, Calhau C, Cassano

A, et al. Polyphenols and human health: a prospectus. Crit Rev Food Sci Nutr. 2011;51(6):524-46.

57. Heber D. Vegetables, fruits and phytoestrogens in the prevention of diseases. J Postgrad Med. 2004;50(2):145-50.

58. Castañeda-Ovando A, Pacheco-Hernández MdL, Páez-Hernández ME, Rodríguez JA, Galán-Vidal CA. Chemical studies of anthocyanins: A review. Food Chem. 2009;113(4):859-71

59. Blando F, Gerardi C, Nicoletti I. Sour cherry (Prunus cerasus L) anthocyanins as ingredients for functional foods. J Biomed Biotechnol. 2004;2004(5):253-8

60. Ramos P, Herrera RM, Moya-Leon M. Anthocyanins: food sources and benefits to consumer's health. Handbook of Anthocyanins 2015. p. 1-22.

61. Tsuda T. Dietary anthocyanin-rich plants: biochemical basis and recent progress in health benefits studies. Mol Nutr Food Res. 2012;56(1):159-70.

62. Kahkonen MP, Heinonen M. Antioxidant activity of anthocyanins and their aglycons. J Agric Food Chem. 2003;51(3):628-33.

63. Yuan B, Imai M, Kikuchi H, Fukushima S, Hazama S, Akaike T, et al. Cytocidal effects of polyphenolic compounds, alone or in combination with, anticancer drugs against cancer cells: potential future application of the combinatory therapy: IntechOpen; 2012. 154-74 p.

64. Farrell NJ, Norris GH, Ryan J, Porter CM, Jiang C, Blesso CN. Black elderberry extract attenuates inflammation and metabolic dysfunction in diet-induced obese mice. Br J Nutr. 2015;114(8):1123-31.

65. Howard LR, Prior RL, Liyanage R, Lay JO. Processing and storage effect on berry polyphenols: challenges and implications for bioactive properties. J Agric Food Chem. 2012;60(27):6678-93.

66. Lohachoompol V, Srzednicki G, Craske J. The change of total anthocyanins in blueberries and their antioxidant effect after drying and freezing. J Biomed Biotechnol. 2004(5):248-52.

67. Ou B, Bosak KN, Brickner PR, Iezzoni DG, Seymour EM. Processed tart cherry products--comparative phytochemical content, in vitro antioxidant capacity and in vitro anti-inflammatory activity. J Food Sci. 2012;77(5):H105-12

68. Prior RL, Wilkes S, Rogers T, Khanal RC, Wu X, Hager TJ, et al. Dietary black raspberry anthocyanins do not alter development of obesity in mice fed an obesogenic high-fat diet. J Agric Food Chem. 2010;58(7):3977-83

69. Kalt W, Ryan DA, Duy JC, Prior RL, Ehlenfeldt MK, Vander Kloet SP. Interspecific variation in anthocyanins, phenolics, and antioxidant capacity among genotypes of highbush and lowbush blueberries (Vaccinium section cyanococcus spp.). J Agric Food Chem. 2001;49(10):4761-7.

70. Wang H, Nair MG, Strasburg GM, Chang YC, Booren AM, Gray JI, et al. Antioxidant and antiinflammatory activities of anthocyanins and their aglycon, cyanidin, from tart cherries. J Nat Prod. 1999;62(2):294-6

71. Skrede G, Wrolstad RE, Durst RW. Changes in anthocyanins and polyphenolics during juice processing of highbush blueberries (Vaccinium corymbosum L.). J Food Sci. 2000;65(2):357-64.

72. Ballistreri G, Continella A, Gentile A, Amenta M, Fabroni S, Rapisarda P. Fruit quality and bioactive compounds relevant to human health of sweet cherry (Prunus avium L.) cultivars grown in Italy. Food Chem. 2013;140(4):630-8

73. Bastos C, Barros L, Duenas M, Calhelha RC, Queiroz MJ, Santos-Buelga C, et al. Chemical characterisation and bioactive properties of Prunus avium L.: the widely studied fruits and the unexplored stems. Food Chem. 2015;173:1045-53.

74. Cao J, Jiang Q, Lin J, Li X, Sun C, Chen K. Physicochemical characterisation of four cherry species (Prunus spp.) grown in China. Food Chem. 2015;173:855-63.

75. de Lima R, Assumpção C, Prestes J, Denadai B. Consumption of cherries as a strategy to attenuate exercise-induced muscle damage and inflammation in humans. Nutr Hosp. 2015;32(5):1885-93.

76. Dimitriou L, Hill JA, Jehnali A, Dunbar J, Brouner J, McHugh MP, et al. Influence of a montmorency cherry juice blend on indices of exercise-induced stress and upper respiratory tract symptoms following marathon running--a pilot investigation. J Int Soc Sports Nutr. 2015;12:22.

77. Gonçalves B, Landbo A-K, Let M, Silva AP, Rosa E, Meyer AS. Storage affects the phenolic profiles and antioxidant activities of cherries (Prunus avium L) on human low-density lipoproteins. J Sci Food Agric. 2004;84(9):1013-20

78. Gonçalves B, Silva AP, Moutinho-Pereira J, Bacelar E, Rosa E, Meyer AS. Effect of ripeness and postharvest storage on the evolution of colour and anthocyanins in cherries (Prunus avium L.). Food Chem. 2007;103(3):976-84.

79. Hayaloglu AA, Demir N. Physicochemical characteristics, antioxidant activity, organic acid and sugar contents of 12 sweet cherry (Prunus avium L.) cultivars grown in Turkey. J Food Sci. 2015;80(3):C564-70

80. Liu Y, Liu X, Zhong F, Tian R, Zhang K, Zhang X, et al. Comparative study of phenolic compounds and antioxidant activity in different species of cherries. J Food Sci. 2011;76(4):C633-8

81. Measham P, Quentin A, MacNair N. Climate, winter chill, and decision-making in sweet cherry production. HortScience. 2013;49:254 - 9.

82. Picariello G, De Vito V, Ferranti P, Paolucci M, Volpe MG. Species- and cultivar-dependent traits of Prunus avium and Prunus cerasus polyphenols. Journal

Food Compost Anal. 2016;45:50-7.

83. Schuller E, Halbwirth H, Mikulic-Petkovsek M, Slatnar A, Veberic R, Forneck A, et al. High concentrations of anthocyanins in genuine cherry-juice of old local Austrian Prunus avium varieties. Food Chem. 2015;173:935-42.

84. Snyder S, Zhao B, Luo T, Kaiser C, Cavender G, Hamilton-Reeves J, et al. Consumption of quercetin and quercetin-containing apple and cherry extracts affects blood glucose concentration, hepatic metabolism, and gene expression patterns in bese C57BL/6J high fat-fed mice. J Nutr. 2016;146(5):1001-7.

85. Usenik V, Fabčič J, Štampar F. Sugars, organic acids, phenolic composition and antioxidant activity of sweet cherry (*Prunus avium L.*). Food Chem. 2008;107(1):185-92.

86. Usenik V, Stampar F, Petkovsek MM, Kastelec D. The effect of fruit size and fruit colour on chemical composition in 'Kordia' sweet cherry (Prunus avium L.). Journal Food Compost Anal. 2015;38:121-30.

87. Wu T, Yin J, Zhang G, Long H, Zheng X. Mulberry and cherry anthocyanin consumption prevents oxidative stress and inflammation in diet-induced obese mice. Mol Nutr Food Res. 2016;60(3):687-94.

88. Bound SA, Close DC, Quentin AG, Measham PF, Whiting MD. Crop Load and Time of Thinning Interact to Affect Fruit Quality in Sweet Cherry. J Agr Sci. 2013;5(8):216-30.

89. Chaovanalikit A, Wrolstad R. Total anthocyanins and total phenolics of fresh and processed cherries and their antioxidant properties. J Food Sci. 2004;69(1):FCT67-FCT72

90. Crupi P, Bleve G, Tufariello M, Corbo F, Clodoveo ML, Tarricone L. Comprehensive identification and quantification of chlorogenic acids in sweet cherry

by tandem mass spectrometry techniques. Journal Food Compost Anal. 2018;73:103-11.

91. de Souza VR, Pereira PA, da Silva TL, de Oliveira Lima LC, Pio R, Queiroz F. Determination of the bioactive compounds, antioxidant activity and chemical composition of Brazilian blackberry, red raspberry, strawberry, blueberry and sweet cherry fruits. Food Chem. 2014;156:362-8

92. González-Gómez D, Lozano M, Fernández-León MF, Bernalte MJ, Ayuso MC, Rodríguez AB. Sweet cherry phytochemicals: Identification and characterization by HPLC-DAD/ESI-MS in six sweet-cherry cultivars grown in Valle del Jerte (Spain). Journal Food Compost Anal. 2010;23(6):533-9.

93. Long L, Whiting MD, Nunez-Elisea R. Sweet cherry cultivars for the fresh market. Oregon: Oregon State University; 2007. Contract No.: PNW604.

94. Mulabagal V, Lang GA, DeWitt DL, Dalavoy SS, Nair MG. Anthocyanin content, lipid peroxidation and cyclooxygenase enzyme inhibitory activities of sweet and sour cherries. J Agric Food Chem. 2009;57(4):1239-46

95. Papapetros S, Louppis A, Kosma I, Kontakos S, Badeka A, Kontominas MG. Characterization and differentiation of botanical and geographical origin of selected popular sweet cherry cultivars grown in Greece. Journal Food Compost Anal. 2018;72:48-56

96. Pappas CS, Takidelli C, Tsantili E, Tarantilis PA, Polissiou MG. Quantitative determination of anthocyanins in three sweet cherry varieties using diffuse reflectance infrared Fourier transform spectroscopy. Journal Food Compost Anal. 2011;24(1):17-21.

97. Petriccione M, De Sanctis F, Pasquariello MS, Mastrobuoni F, Rega P, Scortichini M, et al. The Effect of Chitosan Coating on the Quality and Nutraceutical

Traits of Sweet Cherry During Postharvest Life. Food and Bioprocess Technology. 2014;8(2):394-408.

98. Puniran N, Close DC, Bound SA, Corkrey R, Whiting MD, editors. Fruit colour, size and temperature affect the shelf life of sweet cherry2012: International Society for Horticultural Science (ISHS), Leuven, Belgium.

99. Serradilla MJ, Lozano M, Bernalte MJ, Ayuso MC, López-Corrales M, González-Gómez D. Physicochemical and bioactive properties evolution during ripening of 'Ambrunés' sweet cherry cultivar. LWT - Food Science and Technology. 2011;44(1):199-205.

100. Ilić ZS, Milenković L, Šunić L, Fallik E. Effect of coloured shade-nets on plant leaf parameters and tomato fruit quality. J Sci Food Agric. 2015;95(13):2660-7.

101. Winkel-Shirley B. Biosynthesis of flavonoids and effects of stress. Curr Opin Plant Biol. 2002;5(3):218-23.

102. Agati G, Brunetti C, Di Ferdinando M, Ferrini F, Pollastri S, Tattini M. Functional roles of flavonoids in photoprotection: New evidence, lessons from the past. Plant Physiol Biochem. 2013;72:35-45.

103. Bidart-Bouzat MG, Imeh-Nathaniel A. Global change effects on plant chemical defenses against insect herbivores. J Integr Plant Biol. 2008;50(11):1339-54.

104. Viljevac M, Dugalic K, Jurkovic V, Mihaljevic I, Tomas V, Puskar B, et al. Relation between polyphenols content and skin colour in sour cherry fruits. J Agric Sci. 2012;57(2):57-67.

105. University M. Cherry 2019 [This is a dissection of a cherry showing endocarp, mesocarp & exocarp]. Available from: http://www.cas.miamioh.edu/~meicenrd/Anatomy/Ch11_Secretory/cherry2.jpg.

106. Acquaviva R, Russo A, Galvano F, Galvano G, Barcellona ML, Li Volti G, et

al. Cyanidin and cyanidin 3-O-beta-D -glucoside as DNA cleavage protectors and antioxidants. Cell Biol Toxicol. 2003;19(4):243-52.

107. Cacace J, Mazza G. Optimizatin of extraction of anthocyanins from black currants with aqueous ethanol. J Food Sci. 2003;68(1):240 - 8.

108. Ju Z, Howard L. Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolucs from dried red grape skin. J Agric Food Chem. 2003;51:5207 - 13.

109. Luthria DL. Influence of experimental conditions on the extraction of phenolic compounds from parsley (Petroselinum crispum) flakes using a pressurized liquid extractor. Food Chem. 2008;107(2):745-52.

110. Truong VD, Hu Z, Thompson RL, Yencho GC, Pecota KV. Pressurized liquid extraction and quantification of anthocyanins in purple-fleshed sweet potato genotypes. Journal Food Compost Anal. 2012;26(1-2):96-103.

111. Ghassempour A, Heydari R, Talebpour Z, Fakhari A, Rassouli A, Davies N. Study of new extraction methods for separation of anthocyanins from red grape skins: analysis by HPLC and LC-MS/MS. J Liq Chromatogr Relat Technol. 2008;31(17):2686-703.

112. Sun Y, Liao X, Wang Z, Hu X, Chen F. Optimization of microwave-assisted extraction of anthocyanins in red raspberries and identification of anthocyanin of extracts using high-performance liquid chromatography - Mass spectrometry. Eur Food Res Technol. 2007;225:511-23.

113. Kwon J, Belanger J, Pare J. Optimization of microwave-assisted extraction (MAP) for ginseng components by response surface methodology. J Agric Food Chem. 2003;15(7):1807-10.

114. Espada-Bellido E, Ferreiro-Gonzalez M, Carrera C, Palma M, Barroso C,

Barbero G. Optimisation of the ultrasound-assisted extractino of anthocyanins and total phenolic compounds in mulberry (*Morus nigra)* pulp. Food Chem. 2016;219:23-32.

115. Chen F, Sun Y, Zhao G, Liao X, Hu X, Wu J, et al. Optimization of ultrasound-assisted extraction of anthocyanins in red raspberries and identification of anthocyanins in extract using high-performance liquid chromatography–mass spectrometry. Ultrason Sonochem. 2007;14(6):6292-302.

116. Kajdžanoska M, Petreska J, Stefova M. Comparison of Different Extraction Solvent Mixtures for Characterization of Phenolic Compounds in Strawberries. J Agric Food Chem. 2011;59(10):5272-8.

117. Wojdylo A, Nowicka P, Laskowski P, Oszmianski J. Evaluation of sour cherry (Prunus cerasus L.) fruits for their polyphenol content, antioxidant properties, and nutritional components. J Agric Food Chem. 2014;62(51):12332-45.

118. (SIP) H. Cherry strategic investment plan 2017-2021. Horticulture Innovation Australia; 2017.

119. James P. Australian Cherry Production Guide. South Australia: Tasmanian Institute of Agricultural Research; 2011.

120. Gustavsson J, Cederberg C, Sonesson U, van Otterdijk R, Meybeck A. Global food losses and food waste - extent, causes and prevention. Rome: Food and Agriculture Organizarion of the United Nations (FAO); 2011.

121. Parfitt J, Barthel M, Macnaughton S. Food waste within food supply chains: quantification and potential for change to 2050. Philos Trans R Soc Lond B Biol Sci. 2010;365(1554):3065-81.

122. Berry K. Guide to identify common cherry rot pathogens. Tasmania: Tasmanian Institue of Agriculture (University of Tasmania); 2016. Contract No.:

CY13001.

123. Golding J. Review of international best practice for postharvest managment of sweet cherries. Sydney, Australia: NSW Departmnet of Primary Industries; 2018. Report No.: CY1700.

124. Brownmiller C, Howard LR, Prior RL. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed blueberry products. J Food Sci. 2008;73(5):H72-9.

125. de Ancos B, Ibanez E, Reglero G, Cano MP. Frozen storage effects on anthocyanins and volatile compounds of raspberry fruit. J Agric Food Chem. 2000;48(3):873-9.

126. Hager TJ, Howard LR, Prior RL. Processing and storage effects on monomeric anthocyanins, percent polymeric color, and antioxidant capacity of processed blackberry products. J Agric Food Chem. 2008;56(3):689-95.

127. Johnson AR, Milner JJ, Makowski L. The inflammation highway: metabolism accelerates inflammatory traffic in obesity. Immunological reviews. 2012;249(1):218-38.

128. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003;112(12):1796-808.

129. Lumeng CN, Saltiel AR. Inflammatory links between obesity and metabolic disease. J Clin Invest. 2011;121(6):2111-7.

130. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, et al. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res. 2005;46(11):2347-55.

131. WHO. World health statistics 2018: monitoring health for the SDGs,

sustainable development goals. Geneva: World Health Organisation; 2018.

132. Esposito K, Nappo F, Marfella R, Giugliano G, Giugliano F, Ciotola M, et al. Inflammatory cytokine concentrations are acutely increased by hyperglycemia in humans. Circulation. 2002;106(16):2067-72.

133. Rodgers RJ, Tschop MH, Wilding JP. Anti-obesity drugs: past, present and future. Dis Model Mech. 2012;5(5):621-6.

134. Lean ME, Carraro R, Finer N, Hartvig H, Lindegaard ML, Rossner S, et al. Tolerability of nausea and vomiting and associations with weight loss in a randomized trial of liraglutide in obese, non-diabetic adults. Int J Obes (Lond). 2014;38(5):689-97.

135. Kang JG, Park C-Y. Anti-obesity drugs: A review about their effects and safety. Diabetes Metab. 2012;36(1):13-25.

136. Onakpoya IJ, Heneghan CJ, Aronson JK. Post-marketing withdrawal of anti-obesity medicinal products because of adverse drug reactions: a systematic review. BMC Med. 2016;14(191):1-11.

137. Calabro P, Yeh ET. Intra-abdominal adiposity, inflammation, and cardiovascular risk: new insight into global cardiometabolic risk. Curr Hypertens Rep. 2008;10(1):32-8.

138. Ferrero-Miliani L, Nielsen OH, Andersen PS, Girardin SE. Chronic inflammation: importance of NOD2 and NALP3 in interleukin-1beta generation. Clin Exp Immunol. 2007;147(2):227-35.

139. Ames BN, Shigenaga MK, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci U S A. 1993;90(17):7915-22.

140. Ma Q, Kinneer K. Chemoprotection by phenolic antioxidants. Inhibition of tumor necrosis factor alpha induction in macrophages. J Biol Chem.

2002;277(4):2477-84.

141. Ma Q, Kinneer K, Ye J, Chen BJ. Inhibition of nuclear factor kappaB by phenolic antioxidants: interplay between antioxidant signaling and inflammatory cytokine expression. Mol Pharmacol. 2003;64(2):211-9.

142. Slavin JL, Lloyd B. Health benefits of fruits and vegetables. Adv Nutr. 2012;3(4):506-16.

143. Wang X, Ouyang Y, Liu J, Zhu M, Zhao G, Bao W, et al. Fruit and vegetable consumption and mortality from all causes, cardiovascular disease, and cancer: systematic review and dose-response meta-analysis of prospective cohort studies. BMJ. 2014;349(g4490):1-14.

144. Kaur C, Kapoor HC. Antioxidants in fruits and vegetables – the millennium's health. Int J Food Sci Technol. 2001;36(7):703-25.

145. Robards K, Prenzler PD, Tucker G, Swatsitang P, Glover W. Phenolic compounds and their role in oxidative processes in fruits. Food Chem. 1999;66(4):401-36.

146. Bridle P, Timberlake CF. Anthocyanins as natural food colours—selected aspects. Food Chem. 1997;58(1):103-9.

147. Schlesinger N, Rabinowitz R, Schlesinger M. Pilot studies of cherry juice concentrate for gout flare prophylaxis. J Arthritis. 2012;1(1):1-5.

148. Kelley DS, Adkins Y, Reddy A, Woodhouse LR, Mackey BE, Erickson KL. Sweet bing cherries lower circulating concentrations of markers for chronic inflammatory diseases in healthy humans. J Nutr. 2013;143(3):340-4.

149. Kirakosyan A, Mitchell Seymour E, Noon KR, Urcuyo Llanes DE, Kaufman PB, Warber SL, et al. Interactions of antioxidants isolated from tart cherry (Prunus cerasus) fruits. Food Chem. 2010;122(1):78-83.

150. Ruiz A, Hermosín-Gutiérrez I, Vergara C, von Baer D, Zapata M, Hitschfeld A, et al. Anthocyanin profiles in south Patagonian wild berries by HPLC-DAD-ESI-MS/MS. Food Res Int. 2013;51(2):706-13.

151. Pinelo M, Rubilar M, Jerez M, Sineiro J, Núñez MJ. Effect of solvent, temperature, and solvent-to-solid ratio on the total phenolic content and antiradical activity of extracts from different components of grape pomace. J Agric Food Chem. 2005;53(6):2111-7.

152. Šimunić V, Kovač S, Gaso-Sokac D, Pfannhauser W, Murkovic M. Determination of anthocyanins in four Croatian cultivars of sour cherries (Prunus cerasus). Eur Food Res Technol. 2005;220:575-8

153. Damar I, Eksi A. Antioxidant capacity and anthocyanin profile of sour cherry (Prunus cerasus L.) juice. Food Chem. 2012;135(4):2910-4

154. Gao L, Mazza G. Characterization, quantitation, and distribution of anthocyanins and colorless phenolics in sweet cherries. J Agric Food Chem. 1995;43(2):343-6

155. Moldovan B, David L. Influence of Temperature and Preserving Agents on the Stability of Cornelian Cherries Anthocyanins. Molecules. 2014;19(6):8177-88

156. Serra AT, Duarte RO, Bronze MR, Duarte CMM. Identification of bioactive response in traditional cherries from Portugal. Food Chem. 2011;125(2):318-25

157. Luthria DL. Significance of sample preparation in developing analytical methodologies for accurate estimation of bioactive compounds in functional foods. J Sci Food Agric. 2006;86(14):2266-72.

158. Yılmaz F, Karaaslan M, Vardin H. Optimization of extraction parameters on the isolation of phenolic compounds from sour cherry (Prunus cerasus L.) pomace. J Food Sci Technol. 2014;52(5):2851-9.

159. Kataoka I, Sugiyama A, Beppu K. Role of ultraviolet radiation in accumulation of anthocyanin in berries of 'Gros Colman' grapes (Vitis vinifera L.). J Jpn Soc Hortic Sci. 2003;72(1):1-6.

160. Tarara J, Lee J, Spayd S, Scagel CF. Berry temperature and solar radiation alter acylation, proportion, and concentration of anthocyanin in merlot grapes. Am J Enol Vitic. 2008;59:235-47.

161. Lou X, Janssen H-G, Cramers CA. Parameters affecting the accelerated solvent extraction of polymeric samples. Anal Chem. 1997;69(8):1598-603.

162. Buckow R, Kastell A, Terefe NS, Versteeg C. Pressure and temperature effects on degradation kinetics and storage stability of total anthocyanins in blueberry juice. J Agric Food Chem. 2010;58(18):10076-84.

163. Khanal RC, Howard LR, Prior RL. Effect of heating on the stability of grape and blueberry pomace procyanidins and total anthocyanins. Food Res Int. 2010;43(5):1464-9.

164. Queiroz F, Oliveira C, Pinho O, Ferreira IM. Degradation of anthocyanins and anthocyanidins in blueberry jams/stuffed fish. J Agric Food Chem. 2009;57(22):10712-7.

165. Rajha HN, Darra NE, Hobaika Z, Boussetta N, Vorobiev E, Maroun RG, et al. Extraction of total phenolic compounds, flavonoids, anthocyanins and tannins from grape byproducts by response surface methodology. influence of solid-liquid ratio, particle size, time, temperature and solvent mixtures on the optimization process. Food Nutr Sci. 2014;05(04):397-409.

166. Bridgers EN, Chinn MS, Truong V-D. Extraction of anthocyanins from industrial purple-fleshed sweetpotatoes and enzymatic hydrolysis of residues for fermentable sugars. Ind Crops Prod. 2010;32(3):613 - 20.

167. Revilla E, Ryan J-M, Martín-Ortega G. Comparison of several procedures used for the extraction of anthocyanins from red grapes. J Agric Food Chem. 1998;46(11):4592-7.

168. Rodriguez-Saona L, Wrolstad R. Extraction, isolation, and purification of anthocyanins 2001. 1-11 p.

169. Chan SW, Lee CY, Yap C, Mustapha W, Ho C. Optimisation of extraction conditions for phenolic compounds from limau purut (Citrus hystrix) peels. Int Food Res J. 2009;16(2):203 - 13.

170. Lapornik B, Prosek M, Wondra AG. Comparison of extracts prepared from plant by-products using different solvents and extraction time. J Food Eng. 2005;71(2):214-22.

171. Pennington J, Spungen J. Bowe's and Church's food values of portions commonly used. 19th ed: Wolters Kluwer LWW; 2014.

172. Wadhwa M, Bakshi M. Utilization of fruit and vegetables wastes as livestock. Rome, Italy: Food and Agriculture Organization of the United Nations (FAO); 2013.

173. Choi IS, Kim J-H, Wi SG, Kim KH, Bae H-J. Bioethanol production from mandarin (Citrus unshiu) peel waste using popping pretreatment. Appl Energy. 2013;102:204-10.

174. Patel A, Sindhu DK, Arora N, Singh RP, Pruthi V, Pruthi PA. Biodiesel production from non-edible lignocellulosic biomass of Cassia fistula L. fruit pulp using oleaginous yeast Rhodosporidium kratochvilovae HIMPA1. Bioresour Technol. 2015;197:91-8.

175. Dung TNB, Sen B, Chen C-C, Kumar G, Lin C-Y. Food waste to bioenergy via anaerobic processes. Energy Procedia. 2014;61:307-12.

176. Maitan-Alfenas GP, Visser EM, Guimarães VM. Enzymatic hydrolysis of

lignocellulosic biomass: converting food waste in valuable products. Curr Opin Food Sci. 2015;1:44-9.

177. Lou XF, Nair J, Ho G. Potential for energy generation from anaerobic digestion of food waste in Australia. Waste Manag Res. 2013;31(3):283-94.

178. Pfaltzgraff LA, De bruyn M, Cooper EC, Budarin V, Clark JH. Food waste biomass: a resource for high-value chemicals. Green Chem. 2013;15:307-14.

179. Ayala-Zavala JF, Vega-Vega V, Rosas-Domínguez C, Palafox-Carlos H, Villa-Rodriguez JA, Siddiqui MW, et al. Agro-industrial potential of exotic fruit byproducts as a source of food additives. Food Res Int. 2011;44(7):1866-74.

180. O'Shea N, Arendt EK, Gallagher E. Dietary fibre and phytochemical characteristics of fruit and vegetable by-products and their recent applications as novel ingredients in food products. Innov Food Sci Emerg Technol. 2012;16:1-10.

181. Cortez R, Luna-Vital DA, Margulis D, Gonzalez de Mejia E. Natural Pigments: stabilization methods of anthocyanins for food applications. Compr Rev Food Sci Food Saf. 2017;16(1):180-98.

182. Fernandez-Lopez JA, Angosto JM, Gimenez PJ, Leon G. Thermal stability of selected natural red extracts used as food colorants. Plant Foods Hum Nutr. 2013;68(1):11-7.

183. Sui X, Zhang Y, Zhou W. Bread fortified with anthocyanin-rich extract from black rice as nutraceutical sources: Its quality attributes and in vitro digestibility. Food Chem. 2016;196:910-6.

184. Winter AN, Ross EK, Khatter S, Miller K, Linseman DA. Chemical basis for the disparate neuroprotective effects of the anthocyanins, callistephin and kuromanin, against nitrosative stress. Free Radic Biol Med. 2017;103:23-34.

185. Papaioanou M, Chronopoulou EG, Ciobotari G, Efrose RC, Sfichi-Duke L,

Chatzikonstantinou M, et al. Evaluation of the nutraceutical and cosmeceutical potential of two cultivars of Rubus fruticosus L. under different cultivation conditions. Curr Pharm Biotechnol. 2017;18(11):890-9.

186. Blackhall ML, Berry R, Davies NW, Walls JT. Optimized extraction of anthocyanins from Reid Fruits' Prunus avium 'Lapins' cherries. Food Chem. 2018;256:280-5.

187. Chai W, Morimoto Y, Cooney RV, Franke AA, Shvetsov YB, Le Marchand L, et al. Dietary red and processed meat intake and markers of adiposity and inflammation: The multiethnic cohort ctudy. J Am Coll Nutr 2017;36(5):378-85.

188. Delgado J, Terron Mdel P, Garrido M, Barriga C, Paredes SD, Espino J, et al. Systemic inflammatory load in young and old ringdoves is modulated by consumption of a Jerte Valley cherry-based product. J Med Food. 2012;15(8):707-12.

189. Howatson G, Bell PG, Tallent J, Middleton B, McHugh MP, Ellis J. Effect of tart cherry juice (Prunus cerasus) on melatonin levels and enhanced sleep quality. Eur J Nutr. 2012;51(8):909-16.

190. Bell PG, McHugh MP, Stevenson E, Howatson G. The role of cherries in exercise and health. Scand J Med Sci Sports. 2014;24(3):477-90.

191. Bell PG, Walshe IH, Davison GW, Stevenson E, Howatson G. Montmorency cherries reduce the oxidative stress and inflammatory responses to repeated days high-intensity stochastic cycling. Nutrients. 2014;6(2):829-43.

192. Bowtell JL, Sumners DP, Dyer A, Fox P, Mileva KN. Montmorency cherry juice reduces muscle damage caused by intensive strength exercise. Med Sci Sports Exerc. 2011;43(8):1544-51

193. Connolly DAJ. Efficacy of a tart cherry juice blend in preventing the symptoms

of muscle damage Br J Sports Med. 2006;40(8):679-83.

194. Ellips. Blueberry, date and cherry systems. Netherlands; 2018.

195. Koubaa M, Barba FJ, Grimi N, Mhemdi H, Koubaa W, Boussetta N, et al. Recovery of colorants from red prickly pear peels and pulps enhanced by pulsed electric field and ultrasound. Innov Food Sci Emerg Technol. 2016;37:336-44.

196. Kuden A, Imrak B, Bayazıt S, Comlekcioglu S, Küden A. Chilling requirements of cherries grown under subtropical conditions of Adana. Middle East Journal of Scientific Research. 2012;12:1497-501.

197. Johnson MC, Thomas AL, Greenlief CM. Impact of frozen storage on the anthocyanin and polyphenol contents of american elderberry fruit juice. J Agric Food Chem. 2015;63(23):5653-9.

198. Veberic R, Stampar F, Schmitzer V, Cunja V, Zupan A, Koron D, et al. Changes in the contents of anthocyanins and other compounds in blackberry fruits due to freezing and long-term frozen storage. J Agric Food Chem. 2014;62(29):6926-35.

199. Allaith AA, Ahmed SH, Jafer F. Effect of different thermal treatments and freezing on the antioxidant constituents and activity of two Bahraini date cultivars (Phoenix dactylifera L.). Int J Food Sci Technol. 2012;47(4):783-92.

200. Ketata M, Desjardins Y, Ratti C. Effect of liquid nitrogen pretreatments on osmotic dehydration of blueberries. J Food Eng. 2013;116(1):202-12.

201. Çoklar H, Akbulut M. Effect of sun, oven and freeze-drying on anthocyanins, phenolic compounds and antioxidant activity of black grape (Ekşikara) (Vitis vinifera L.). S Afr J Enol Vitic. 2017;38(2):264-72.

202. Celli GB, Ghanem A, Brooks MS-L. Influence of freezing process and frozen storage on the quality of fruits and fruit products. Food Rev Int. 2015;32(3):280-304.

203. Karaklajić-Stajić Ž, Nikolić M, Tomić J, Pešaković M, Miletić R. Influence of ripeness stage on the rate of pigment degradation in blackberry fruits during frozen storage. Eur J Hortic Sci. 2017;82(4):198-203.

204. Ellulu MS, Patimah I, Khaza'ai H, Rahmat A, Abed Y. Obesity and inflammation: the linking mechanism and the complications. Arch Med Sci. 2017;13(4):851-63.

205. Herder C, Schneitler S, Rathmann W, Haastert B, Schneitler H, Winkler H, et al. Low-grade inflammation, obesity, and insulin resistance in adolescents. J Clin Endocrinol Metab. 2007;92(12):4569-74.

206. Manco M, Putignani L, Bottazzo GF. Gut microbiota, lipopolysaccharides, and innate immunity in the pathogenesis of obesity and cardiovascular risk. Endocr Rev. 2010;31(6):817-44.

207. Rao LN, Ponnusamy T, Philip S, Mukhopadhyay R, Kakkar VV, Mundkur L. Hypercholesterolemia induced immune response and inflammation on progression of atherosclerosis in apob(tm2Sgy) ldlr(tm1Her)/J mice. Lipids. 2015;50(8):785-97.

208. Collaborators GBDO, Afshin A, Forouzanfar MH, Reitsma MB, Sur P, Estep K, et al. Health effects of overweight and obesity in 195 countries over 25 years. N Engl J Med. 2017;377(1):13-27.

209. Allison DB, Gadde KM, Garvey WT, Peterson CA, Schwiers ML, Najarian T, et al. Controlled-release phentermine/topiramate in severely obese adults: a randomized controlled trial (EQUIP). Obesity. 2011;20(2):330-42.

210. Neoh SL, Sumithran P, Haywood CJ, Houlihan CA, Lee FT, Proietto J. Combination phentermine and topiramate for weight maintenance: the first Australian experience. Med J Aust. 2014;201(4):224-6.

211. Padwal R, Kezouh A, Levine M, Etminan M. Long-term persistence with

orlistat and sibutramine in a population-based cohort. Int J Obes (Lond). 2007;31(10):1567-70.

212. Wu T, Tang Q, Gao Z, Yu Z, Song H, Zheng X, et al. Blueberry and mulberry juice prevent obesity development in C57BL/6 mice. PLoS One. 2013;8(10):e77585.

213. Guo S, Qiu P, Xu G, Wu X, Dong P, Yang G, et al. Synergistic anti-inflammatory effects of nobiletin and sulforaphane in lipopolysaccharide-stimulated RAW 264.7 cells. J Agric Food Chem. 2012;60(9):2157-64.

214. Seymour EM, Urcuyo-Llanes D, Bolling SF, Bennink MR. Tart cherry intake reduces plasma and tissue inflammation in obesity-prone rats. FASEB. 2010;24(1_supplement):335.

215. Suh JH, Romain C, Gonzalez-Barrio R, Cristol JP, Teissedre PL, Crozier A, et al. Raspberry juice consumption, oxidative stress and reduction of atherosclerosis risk factors in hypercholesterolemic golden Syrian hamsters. Food Funct. 2011;2(7):400-5.

216. Figueira ME, Camara MB, Direito R, Rocha J, Serra AT, Duarte CM, et al. Chemical characterization of a red raspberry fruit extract and evaluation of its pharmacological effects in experimental models of acute inflammation and collagen-induced arthritis. Food Funct. 2014;5(12):3241-51.

217. Vendrame S, Daugherty A, Kristo AS, Riso P, Klimis-Zacas D. Wild blueberry (Vaccinium angustifolium) consumption improves inflammatory status in the obese Zucker rat model of the metabolic syndrome. J Nutr Biochem. 2013;24(8):1508-12.

218. NHMRC. Australian code for the care and use of animals for scientific purposes. NHMRC, editor: NHMRC; 2013.

219. Kim DH, Sandoval D, Reed JA, Matter EK, Tolod EG, Woods SC, et al. The role of GM-CSF in adipose tissue inflammation. Am J Physiol Endocrinol Metab.

2008;295(5):E1038-46.

220. Wei X, Wang D, Yang Y, Xia M, Li D, Li G, et al. Cyanidin-3-O-beta-glucoside improves obesity and triglyceride metabolism in KK-Ay mice by regulating lipoprotein lipase activity. J Sci Food Agric. 2011;91(6):1006-13.

221. Pirie AD, Davies NW, Ahuja KD, Adams MJ, Shing CM, Narkowicz C, et al. Hypolipidaemic effect of crude extract from Carpobrotus rossii (pigface) in healthy rats. Food Chem Toxicol. 2014;66:134-9.

222. Jiménez-Ferrer E, Alarcón-Alonso J, Aguilar-Rojas A, Zamilpa A, Jiménez-Ferrer C I, Tortoriello J, et al. Diuretic effect of compounds from Hibiscus sabdariffa by modulation of the aldosterone activity. Planta Medica. 2012;78(18):1893-8.

223. Hooman N, Mojab F, Nickavar B, Pouryousefi-Kermani P. Diuretic effect of powdered Cerasus avium (cherry) tails on healty volunteers. Pak J Pharm Sci. 2009;22(4):381-3.

224. Tsuda T. Recent progress in anti-obesity and anti-diabetes effect of berries. Antioxidants (Basel). 2016;5(2).

225. Ju R, Zheng S, Luo H, Wang C, Duan L, Sheng Y, et al. Purple sweet potato attenuate weight gain in high fat diet induced obese mice. J Food Sci. 2017;82(3):787-93.

Appendices

Appendix 1: Detail of cultivars grown in Tasmania that were discussed as part of this thesis

Common name	Full species name	Genus	Species	Hybrid parentage	Family name	Cultivar origin
Lapins	Prunus avium L.	Prunus	Sweet cherry	Van x Stella	Rosaceae	Canada
Kordia	Prunus avium L.	Prunus	Sweet cherry	Lambert x John Innes Seedling 2420	Rosaceae	Czech Republic
Sylvia	Prunus avium L.	Prunus	Sweet cherry	Van x Compact Lambert	Rosaceae	Canada
Van	Prunus avium L.	Prunus	Sweet cherry	Empress Eugenie x open pollination	Rosaceae	Canada
Stella	Prunus avium L.	Prunus	Sweet cherry	Lambert x John Innes Seedling 2420	Rosaceae	Canada
Sweet Georgia	Prunus avium L.	Prunus	Sweet cherry	Lapin mutation	Rosaceae	Australia
Regina	Prunus avium L.	Prunus	Sweet cherry	Schneiders spate knorpelkirsche' × 'Rube'	Rosaceae	Germany
Simone	Prunus avium L.	Prunus	Sweet cherry	Stella x Van	Rosaceae	Canada
Sweetheart	Prunus avium L.	Prunus	Sweet cherry	Van x Newstar	Rosaceae	Canada
Fertard	Prunus avium L.	Prunus	Sweet cherry	Fercer mutation	Rosaceae	France

Appendix 2: Table comparing anthocyanin content of cultivars from the literature

Sweet Cherry Cultivars (Prunus avium L.)			
Common name	**Anthocyanin (mg/100g FW)**	**Common name**	**Anthocyanin (mg/100g FW)**
0-900 Ziraat	25	Moreau	32
5-106	147	Mpakirtzeika	310
Ambrunes	39 - 70	Napoleona	13 - 14
Badascony	14	Navalinda	123
Belge	29	Noire de Meched	11
Benton	74	Pico Colorado	29
Bing	26 - 225	Pico Negor Limon	45
Black Gold	65	Kordia	184
Black pearl	70	Puntalazzese	58
Blaze Star	62 - 85	Rainier	21
Burlat	13 - 165	Rainier	0.5 - 4
CAB	120	Regina	159
Caihong	20	Royal Ann (Napoleon)	1
Colt	89	Saco	296
Dalbasti	20	Santina	98
Donnantonio	33	Skeena	200 - 390
Ducignola Nera	40	Skeena Edessa	200
Durona di Cesena	17	Skeena Kozani	300
Early Star	47	Summit	29
Fercer	10	Sunburst	25
Fernier	16	Sweet Early	71
Ferprime	10	Early Van Compact	9
Ferrador	1	Sweetheart	19
Ferrovia	27	Sylvia	11
Garnet	653	Tieton	126
Gabbaladri	6	Toscana	29
Genovese	25	Ulster	292
Giorgia	33	Van	9 - 251
Glacier	87	Vesseaux	15
Grace Star	27	Vigred	15
Hardy Giant	7	Vista	15
Hedelfingen	34	Voguee	175
Hongdeng	143	Zaodaguo	119
Hongyan	20	Zio Peppino	8
Kiona	85		
Kordia	184 - 250		
Kristin	82		
Lala Star	13		
Lambert	15		
Lapins	4 - 372		
Maring	151		
Maiolina Grappolo	37		
Maredda	94		
Merton Late	26		
Minnulara	28		

Appendix 2 continued

Sour Cherry Cultivars (Prunus cerasus or Prunus pseudocerasus)			
Common name	**Anthocyanin (mg/100g FW)**	**Common Name**	**Anthocyanin (mg/100g FW)**
Aarslev 1803	285	Duan Bing	7
Aarslev 2403	266	Black peel	6
Aarslev 2504	176		
Aarslev 2510	256		
Amarena Mattarello	80		
Aode	82		
Balaton	5		
Bofa	228		
Brigitte x Bottermo	272		
Capuli	0.68 mg/g DW **		
Cigganymeggy 7	122		
Dana x 1	146		
Erdi Bottermo	65		
Erdi Bottermo	98		
Fanal	250		
Favorit	40		
Gerema	95		
Heimanns Rubin 4	237		
K27/2	81		
Kelleris 16	60		
Lutovka	102		
M7	109		
Montmorency	2 - 8.7		
Nadwislanka	183		
Nefris	202		
Oblachinska Holo	105		
Pernilla	133		
Recta	214		
Safir	200		
Skyggemorel Hannover	61		
Stevnsbaer, Birgitte	175		
Stevnsbaer, PH	185		
Stevnsbaer, Viki	178		
Sumadinka	131		
Surefire	21		
Tiki	264		
Ungarische Traubige	85		
Visciola Ninmo	28		
Visciola Sannicandro	75		
Vytenu Star	92		
Zagarvysne	83		
Zigeunerkirschen	107		

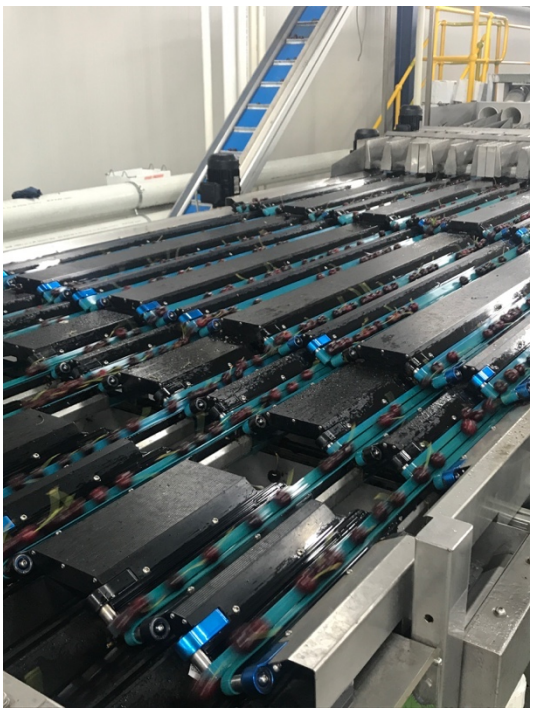
Appendix 2: Pictorial demonstration of cherry sorting process

Cherries arriving from orchard

Loaded onto conveyor for washing

Two step washing process

Appendix 3 - continued



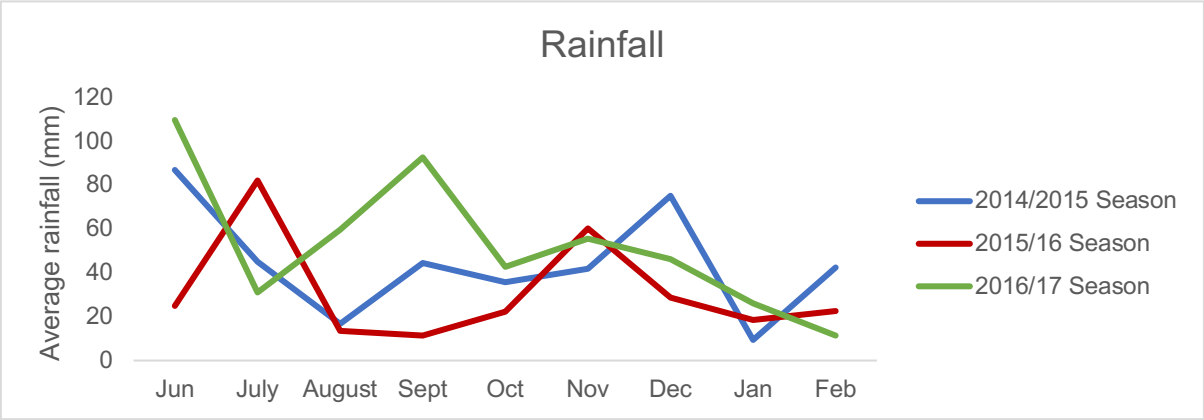
Optical grading using Airjet machine with Ellips software

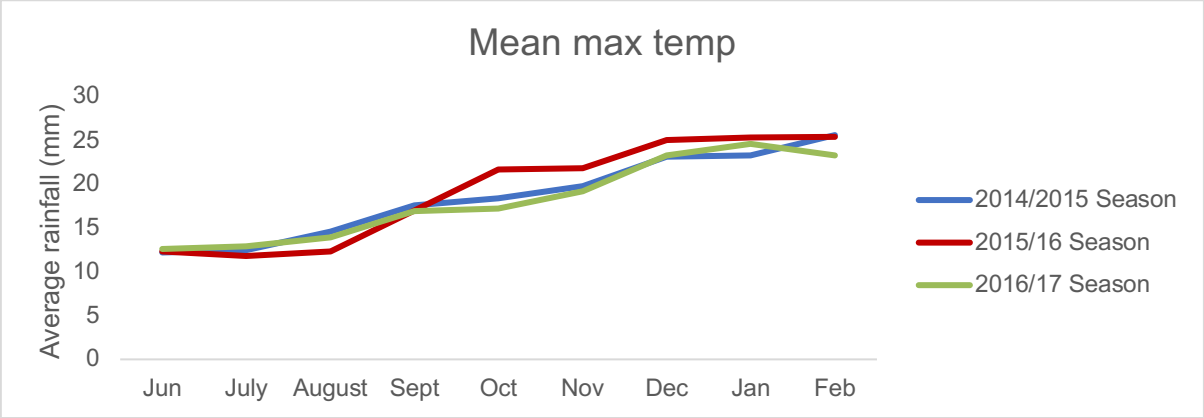
Premium cherries undergo final hand sort before being boxed

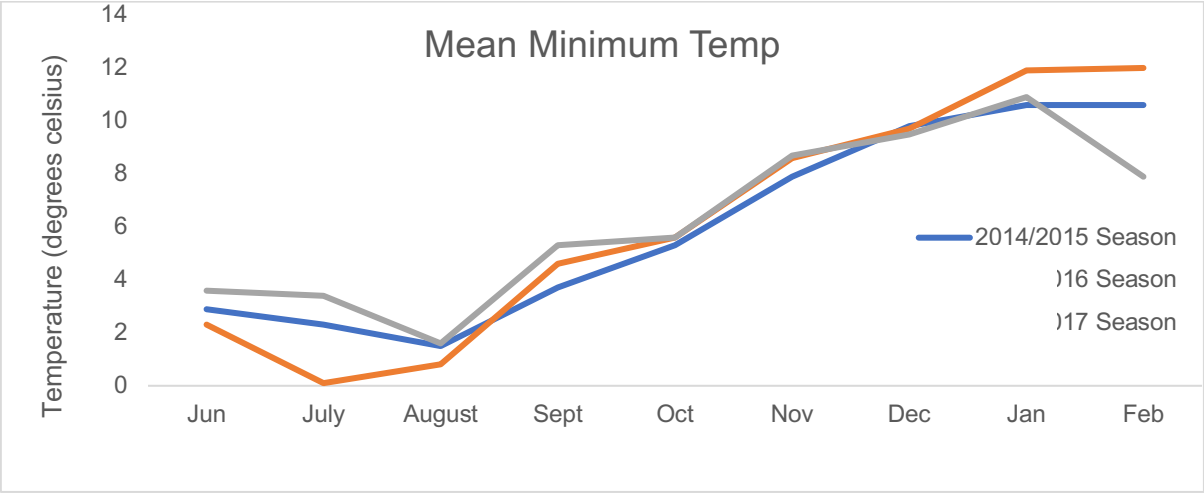
Appendix 3 - continued

Premium grade Lapin cherries boxed ready for market

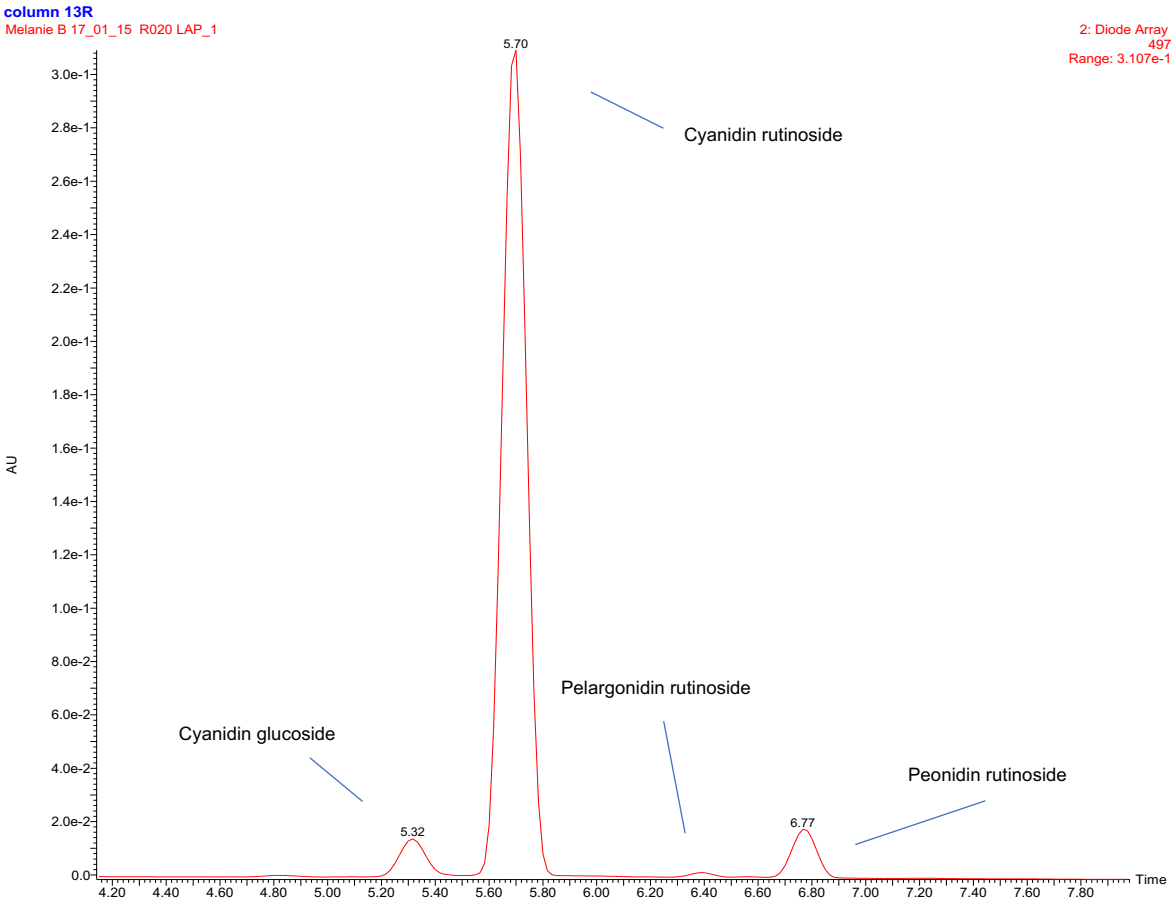
Appendix 4: Retrospective weather data across seasons



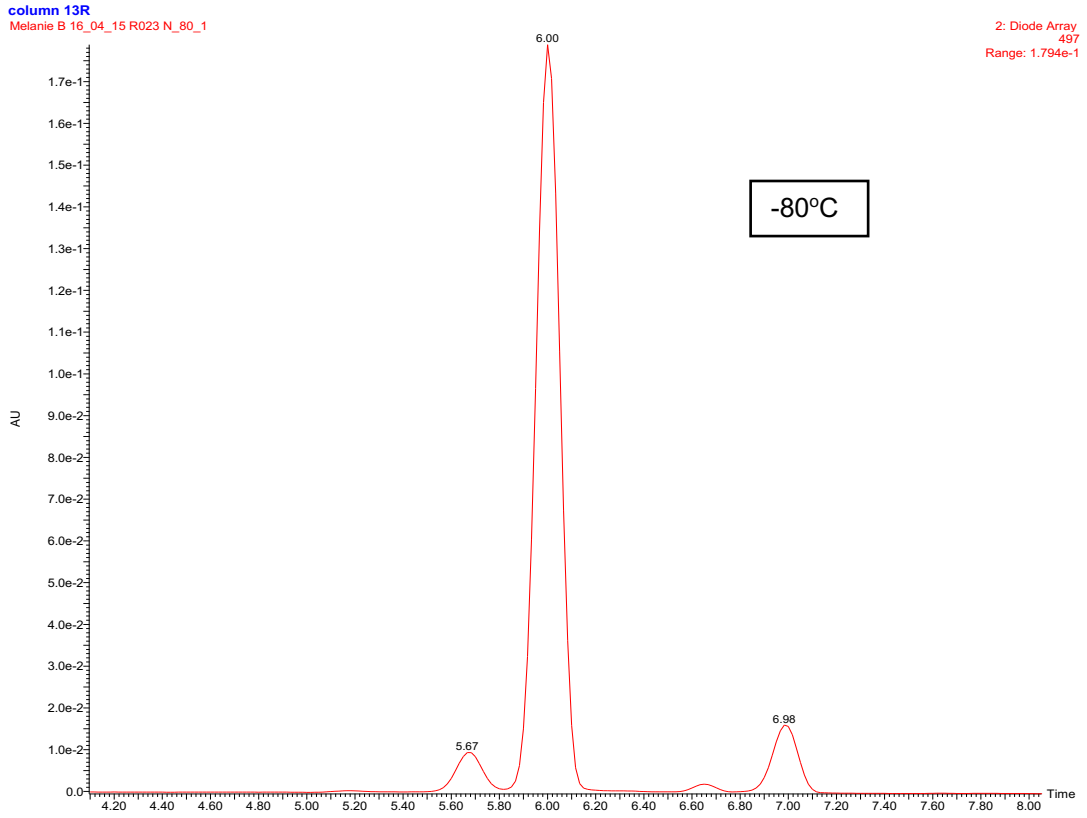


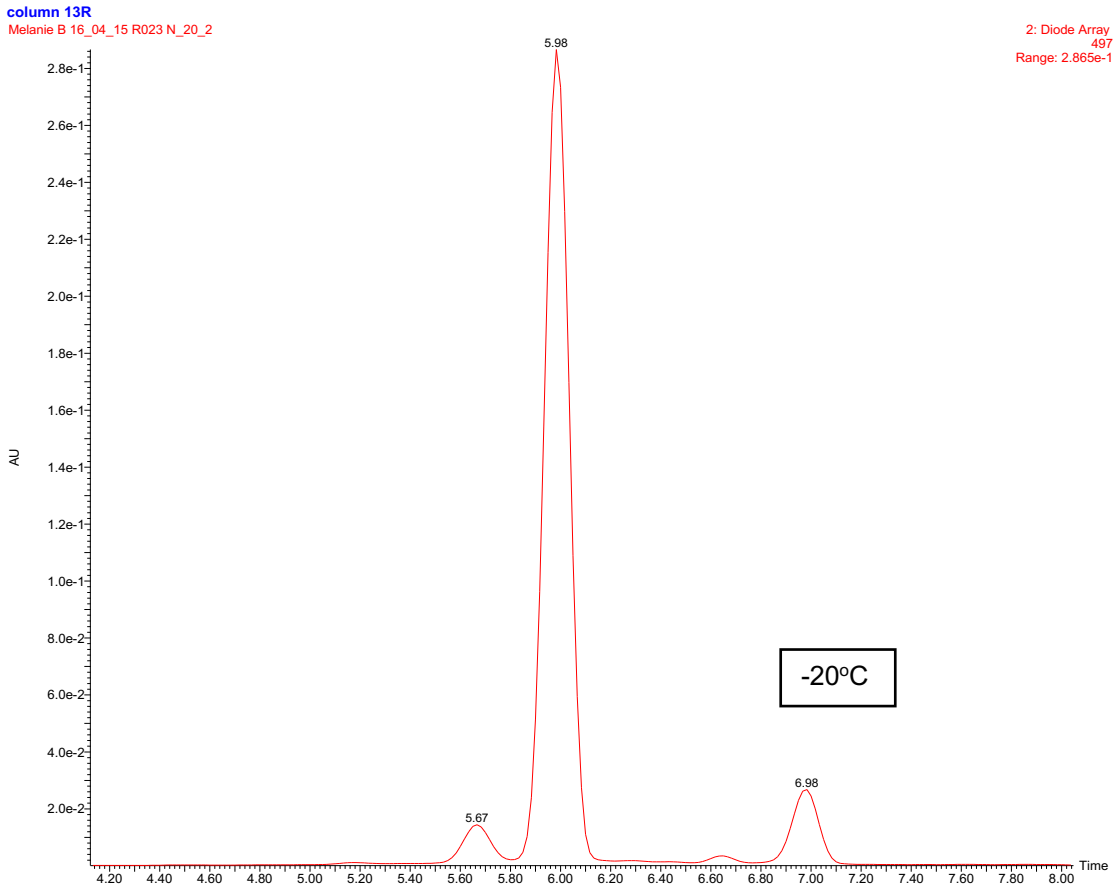


Appendix 5: (a) Storage chromatograms 0 months post-harvest

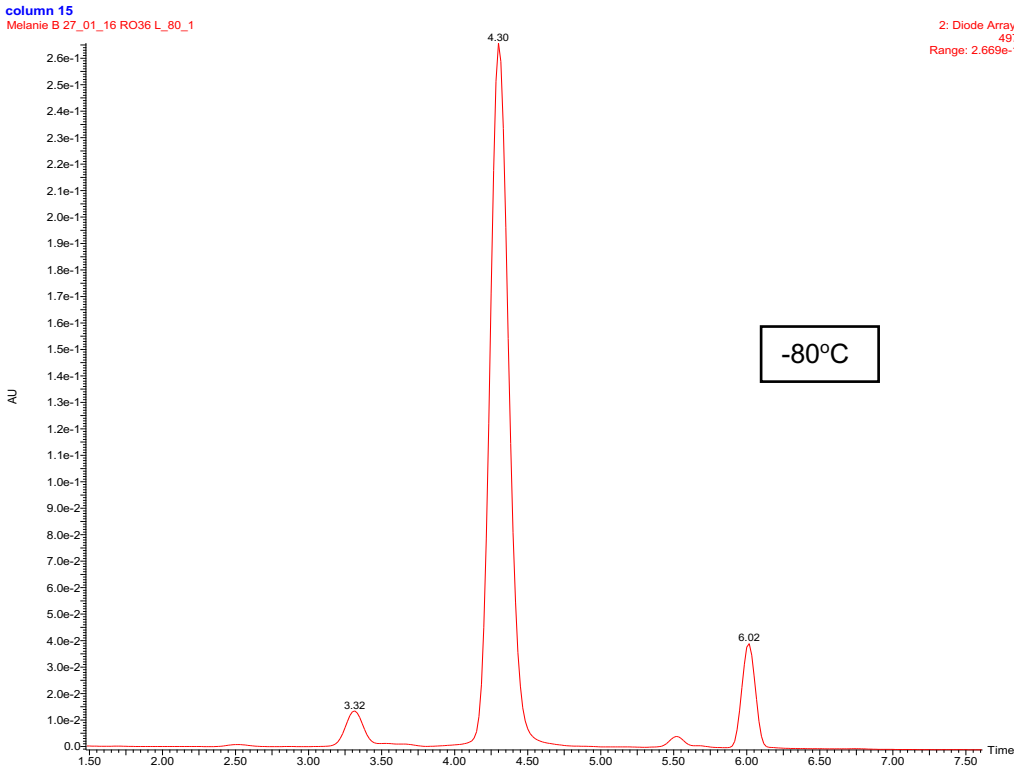


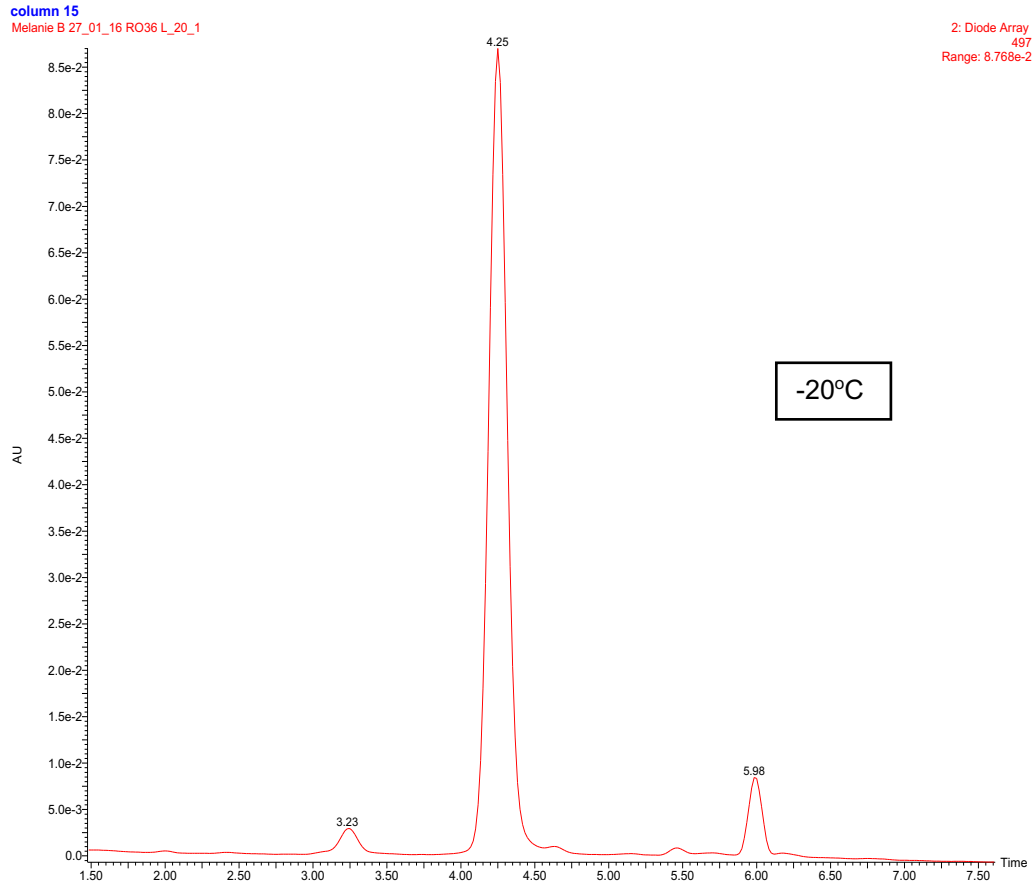
Appendix 5 (b): Storage chromatograms 3 months post-harvest



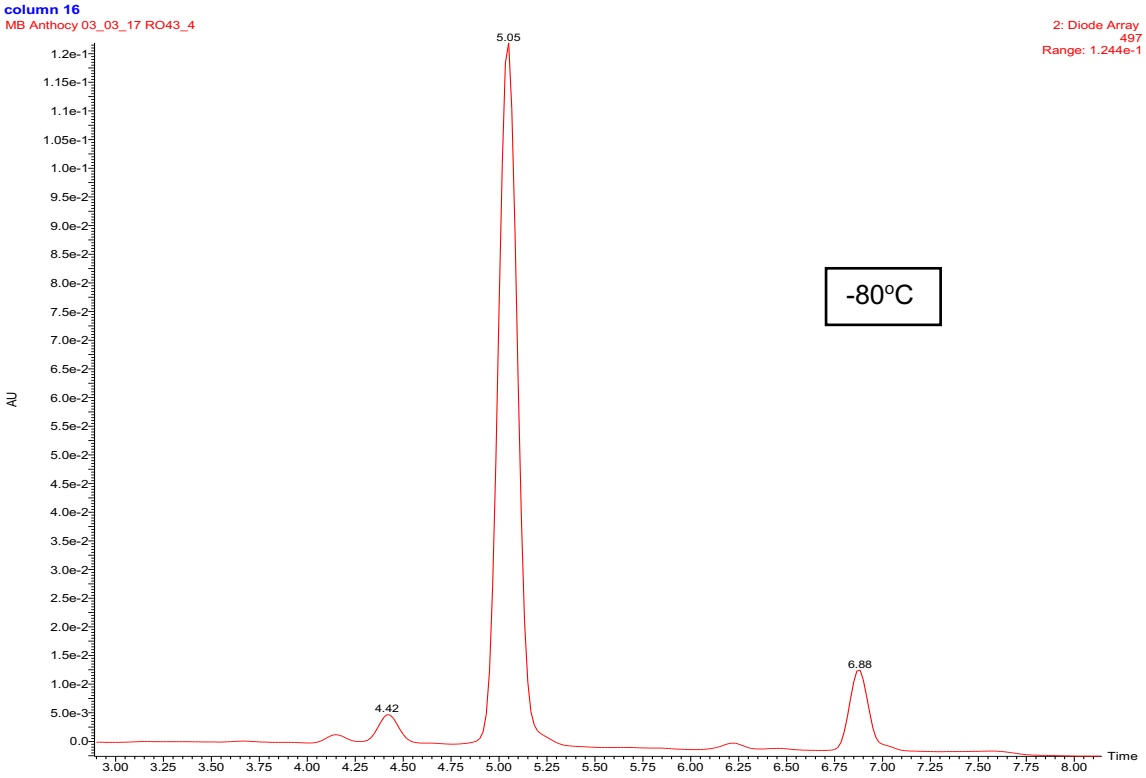


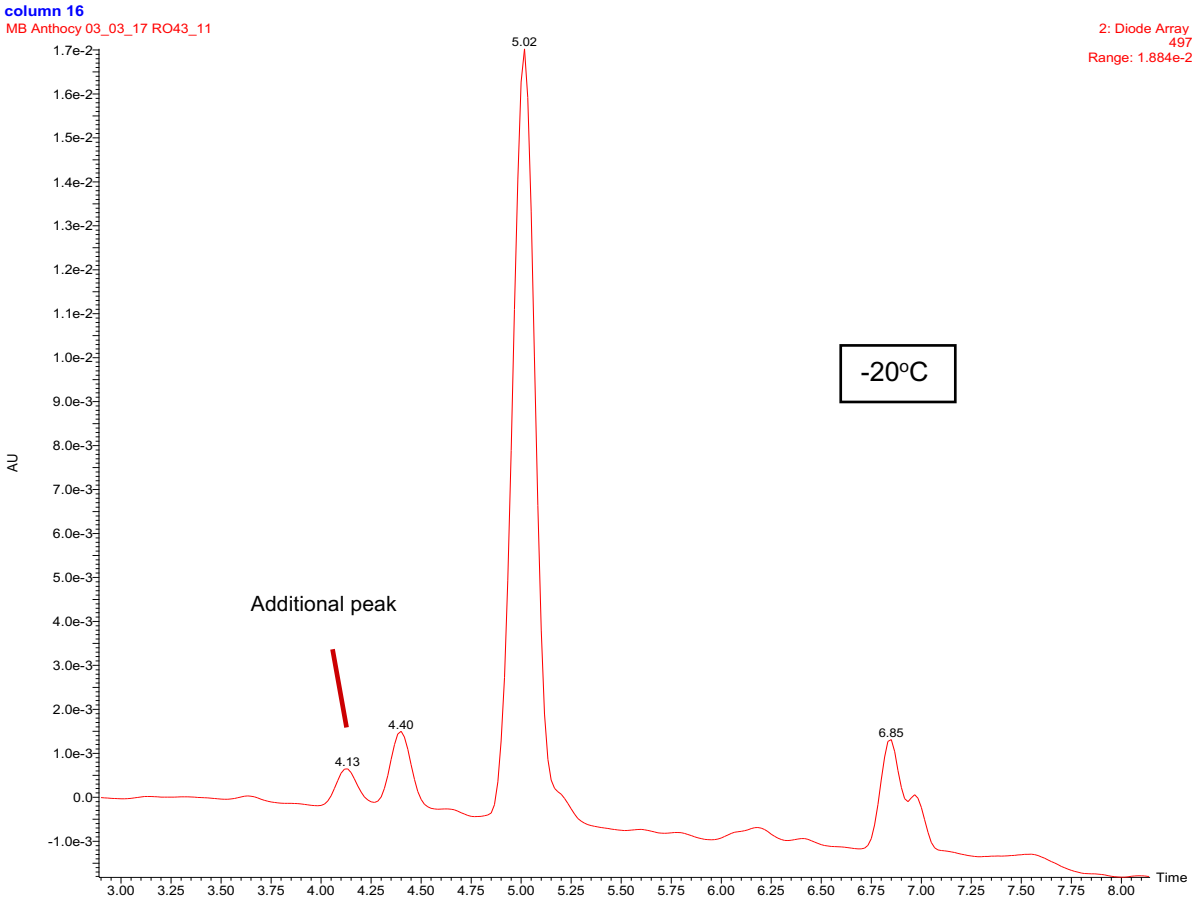
Appendix 5 (c): Storage chromatograms 12 months post-harvest



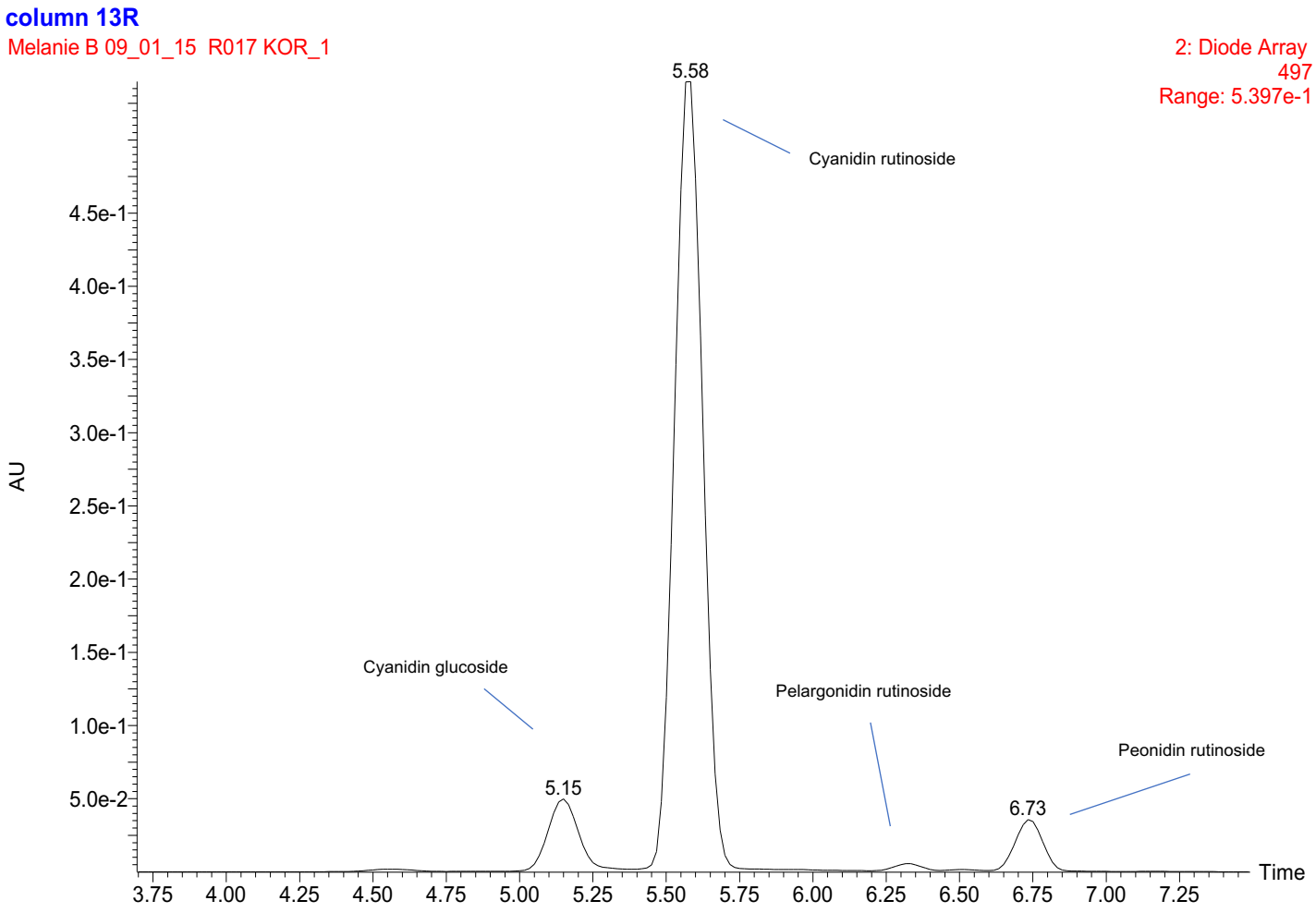


Appendix 5 (d) Storage chromatograms 24 months post- harvest

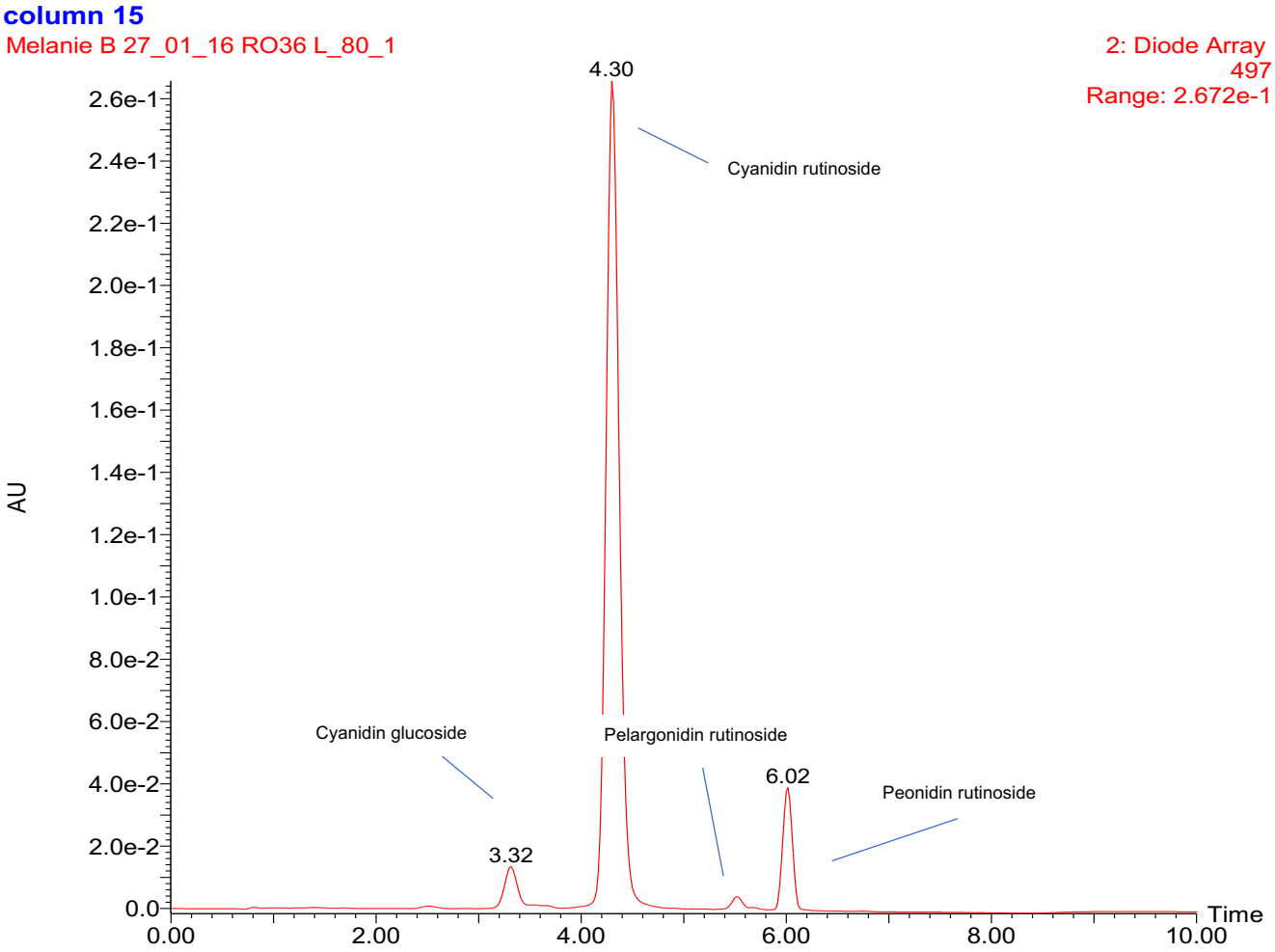




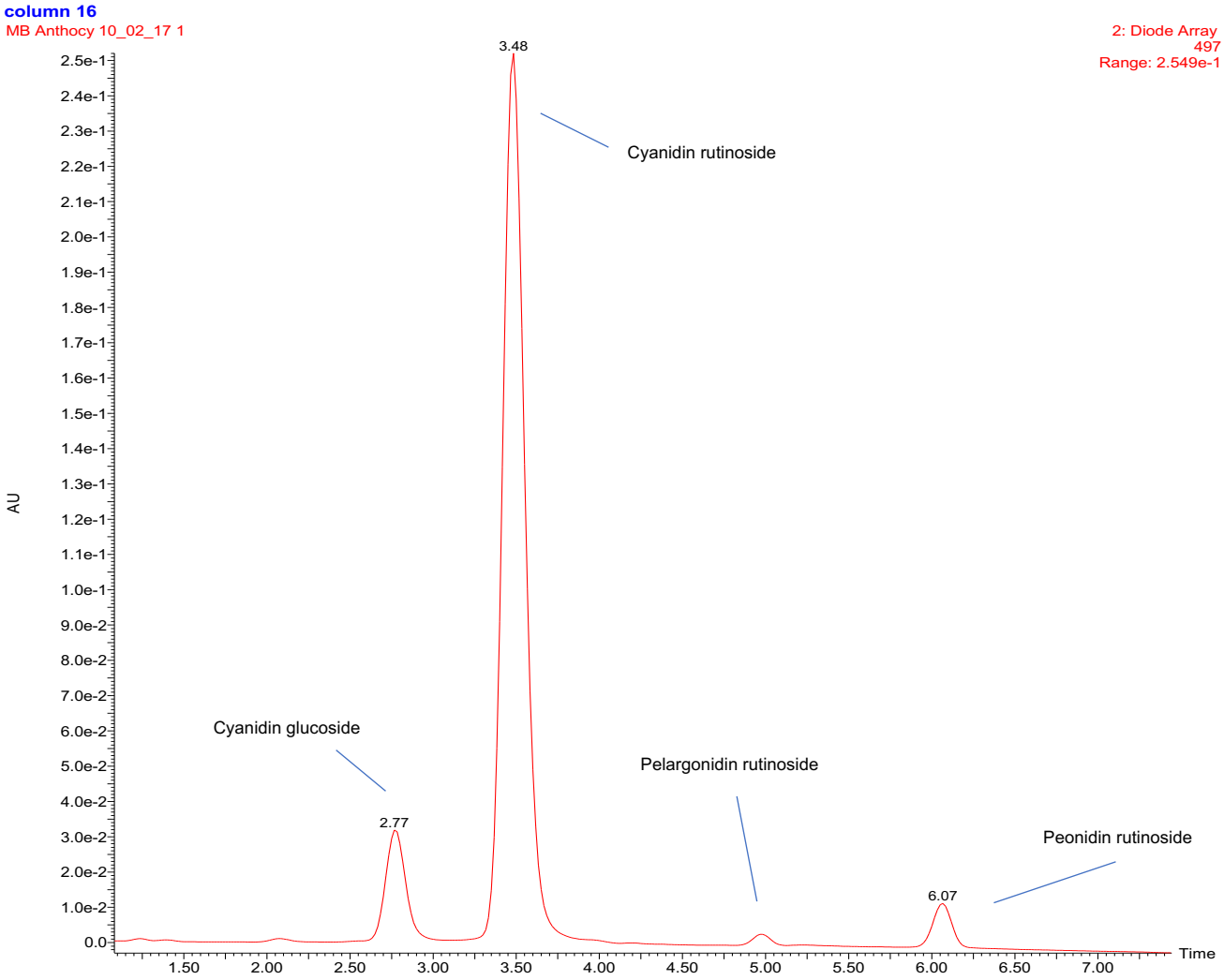
Appendix 6: Chromatograms showing consistency of main anthocyanins identified in the sweet cherry anthocyanin extract (extract taken from whole cherry fruit).



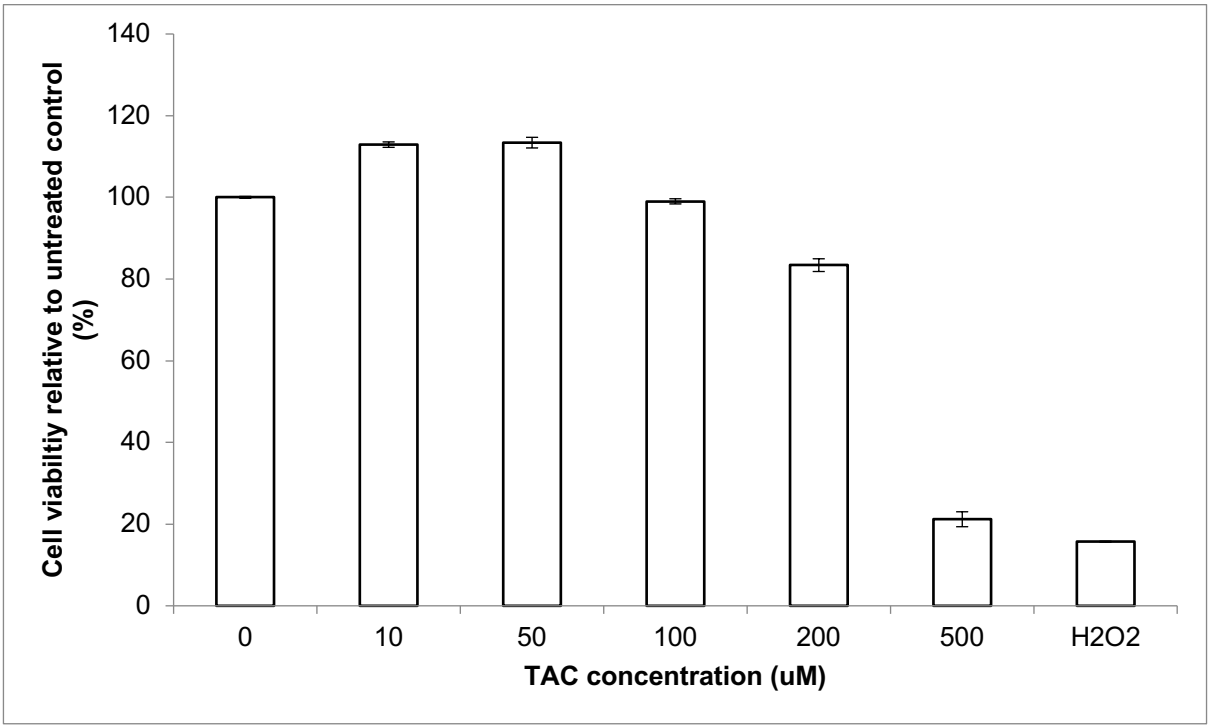
Appendix 6 continued



Appendix 6 continued



Appendix 7: Cell viability assay showing cell survival at different concentrations of anthocyanin



Appendix 8: Poster at joint meeting of Nutrition Society of New Zealand & Nutrition Society of Australia, Wellington, NZ, 2015

UNIVERSITY of TASMANIA

'Physiological' temperature found to be prime for extracting sweet cherry anthocyanins

Melanie L Blackhall[1], Rachael Berry[1], Noel W Davies[2], Justin T Walls[1]

[1]School of Medicine, University of Tasmania [2]Central Science Laboratory, University of Tasmania

Introduction

Anthocyanins are bioactive compounds found in many fruits and vegetables, and have been a recent focus of research due to their high antioxidant activity. Cherries are known to be a rich source of anthocyanins, however evidence regarding the anthocyanin content of sweet cherry (*Prunus avium* L.) cultivars is highly inconsistent. It is unlikely that such large variations can be ascribed to differences in geographical location and environment alone, and are more likely explained by different post-harvest factors, including storage conditions and extraction methods. Extraction optimisation not only results in a greater yield of the compound, but also allows more accurate comparisons to be made between different cultivars of a species. A variety of methods for anthocyanin extraction have been described, including the maceration method, which is an attractive option for smaller laboratories as it requires no specialist equipment. While optimisation of anthocyanin extraction has been described for a variety of fruits, extraction from whole sweet cherries has not been comprehensively investigated.

Methods

This study determined the optimal parameters for the extraction of anthocyanins via maceration of the edible portion of *Prunun avium* 'Lapins' cherries. Extractions with one or two independent variables were conducted to investigate the effect of time and temperature of extraction, solvent to solid ratio, solvent type and solvent concentration on extraction efficiency. Individual anthocyanins were separated via UPLC, with peak assignment based on matching UV-visible spectra with retention times from previous in-house studies and published cherry anthocyanins [1]. The optimal condition for each parameter was based on total anthocyanin content (TAC), calculated by summation of UPLC-derived peak areas, expressed as mg of cyanidin-3-glucoside equivalents per 100g fresh weights.

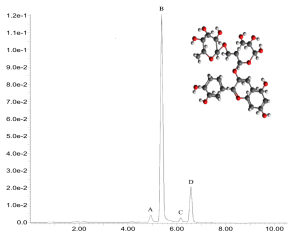


Figure 1 – Extracts were analysed via UPLC to determine the anthocyanin content and profile of *Prunus avium* Lapins cherries. A – cyanidin-3-glucoside; B – cyanidin-3-rutinoside; C – pelargonidin-3-rutinoside; D – peonidin-3-rutinoside.
Inset – Chemical structure of cyanidin-rutinoside – the most abundant anthocyanin in Reid Fruits Lapins cherries

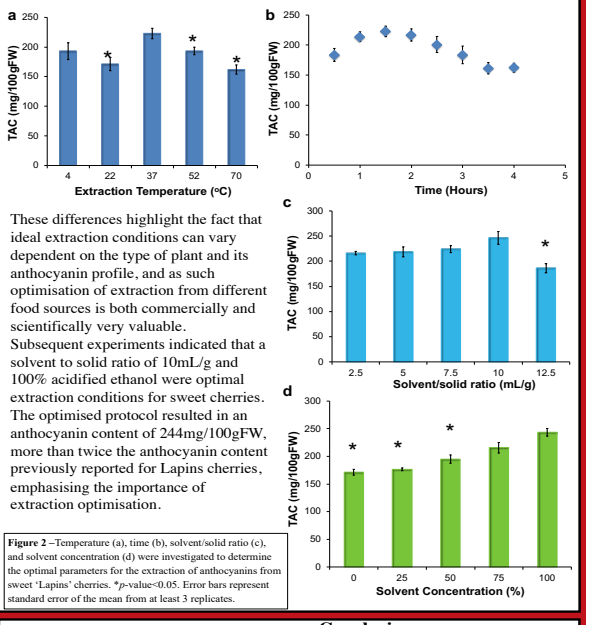
Four different anthocyanins were detected in all extracts (Figure 1). The major anthocyanin identified was cyanidin-3-rutinoside, and accounted for approximately 80% of anthocyanin equivalents in all samples. Peonidin-3-rutinoside was identified as the second major pigment, accounting for approximately 15% of anthocyanin equivalents, whilst two minor peaks, identified as cyanidin-3-glucoside and pelargonidin-3-rutinoside, accounted for the remaining 5% of detected anthocyanins.

Acknowledgements

This work could not have been completed without the support of Reid Fruits and Essential Oils of Tasmania. The researchers would also like to thank Associate Professor John Ross and Dr David Nichols.

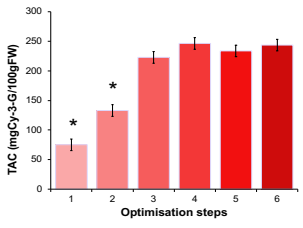
Results & Discussion

Time and temperature were investigated simultaneously to reveal any interaction between the two parameters. Cherries were extracted for between 30 minutes and 24 hours at 4, 22, 37, 52 and 70ºC. The highest anthocyanin yield was observed following extraction at 37ºC (Figure 2a). TAC reached 222.82 ± 8.88mg/100gFW at 1.5 hours, after which further increases in extraction time resulted in a decrease in yield. Samples extracted for 1.5 hours at 37ºC had significantly higher anthocyanin content than those extracted for 1.5 hours at 22ºC ($p < 0.005$) or 70ºC ($p < 0.001$) (Figure 2b). Anthocyanin content was also lower in the 4ºC and 56ºC samples, although not significantly. In contrast, temperatures as high as 93ºC and 75ºC have been reported as optimal in studies investigating extraction from grape and sour cherry pomace, respectively [2,3].



These differences highlight the fact that ideal extraction conditions can vary dependent on the type of plant and its anthocyanin profile, and as such optimisation of extraction from different food sources is both commercially and scientifically very valuable. Subsequent experiments indicated that a solvent to solid ratio of 10mL/g and 100% acidified ethanol were optimal extraction conditions for sweet cherries. The optimised protocol resulted in an anthocyanin content of 244mg/100gFW, more than twice the anthocyanin content previously reported for Lapins cherries, emphasising the importance of extraction optimisation.

Figure 2 – Temperature (a), time (b), solvent/solid ratio (c), and solvent concentration (d) were investigated to determine the optimal parameters for the extraction of anthocyanins from sweet 'Lapins' cherries. **p*-value<0.05. Error bars represent standard error of the mean from at least 3 replicates.



Conclusions

The TAC of 'Lapins' cherries determined using the optimised protocol is more than twice that previously reported. Results from our study contradict previous anthocyanin extraction research, which advocates the use of either low temperature or short, high temperature extractions to maximise yield. The novel finding that extraction at physiological temperature results in the highest TAC is of significance when considering translation to *in vivo* applications.

Contact Us
Faculty of Health
For further information contact
Phone: +61 3 62267638
Email: Melanie.Blackhall@utas.edu.au
www.utas.edu.au/health

CRICOS: 00586B

References
1. Usenik, V., Fabčič, J., & Štampar, F. (2008). Sugars, organic acids, phenolic composition and antioxidant activity of sweet cherry (Prunus avium L.). *Food Chemistry*, 107(1), 185-92
2. Rajha, H. N., Darra, El, N., Hobaika, Z., Boussetta, N., Vorobiev, E., Maroun, R. G., & Louka, N. (2014). Extraction of Total Phenolic Compounds, Flavonoids, Anthocyanins and Tannins from Grape Byproducts by Response Surface Methodology. Influence of Solid-Liquid Ratio, Particle Size, Time, Temperature and Solvent Mixtures on the Optimization Process. *Food and Nutrition Sciences*, 5, 351-360
3. Yılmaz, F. M., Karaaslan, M., & Vardin, H. (2015). Optimization of extraction parameters on the isolation of phenolic compounds from sour cherry (Prunus cerasus L.) pomace. *Journal of Food Science and Technology*, 52(5), 2851-9

Appendix 9: Poster at Nutrition Society of Australia, Annual Scientific Meeting 2018

UNIVERSITY of TASMANIA

In vitro investigation of sweet cherry extract

Blackhall, ML[1], Berry, R[1], Ahuja[1], KDK, Geraghty, DP[1] and Walls, JT[2]

[1] College of Health and Medicine, University of Tasmania, [2] University College, University of Tasmania

Introduction

Inflammation is acknowledged as the precursor to many diseases[1] with water-soluble flavonoids, anthocyanins, implicated in reducing this disease risk. Anthocyanins are bioactive compounds found in many fruits and vegetables, and have been a recent focus of research due to their high antioxidant activity. Sweet cherries (*Prunus avium* L.) are known to be a rich source of anthocyanins[2].

The **aim** of the present study was to determine whether sweet cherry anthocyanins (SCA) can reduce the release of inflammatory mediators from lipopolysaccharide (LPS)-stimulated RAW267.4 macrophages.

Methods

RAW264.7 murine macrophages were cultured in RPMI media, supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) and 2% penicillin-streptomycin stabilised solution.

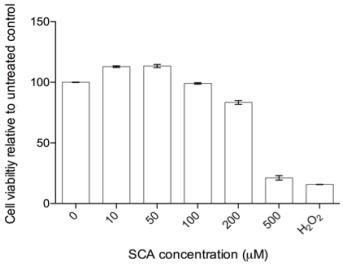
The cells were first pre-treated with SCA (0 – 500μM) for 24 hours at 37°C (with H_2O_2 the blank) to understand the impact of concentration on cell toxicity. H2O2 was used as blank during assay.

To determine impact of pre-treatment with SCA on markers of inflammation, the cells were stimulated with LPS (1μg/mL2)[3]. After 24 hours, cells were harvested via scraping and the suspension centrifuged. Both the supernatant and cell pellet were collected and stored at -80°C until ELISA analysis.

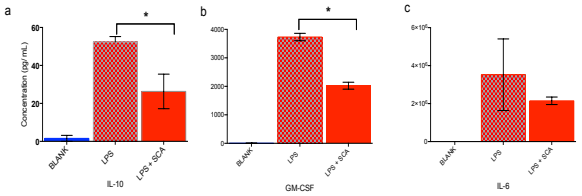
Concentrations of the inflammatory mediators interleukin 6 (IL-6), interleukin 10 (IL-10) and granulocyte macrophage colony stimulating factor (GM-CSF) in the culture media were measured using ELISA.

Results

Following treatment with 10, 50 and 100 μM SCA, the viability of the cells were 113 ± 0.65, 113 ± 1.31 and 99 ± 0.63%, respectively, as compared to the untreated control (100 ± 0.24%). These results indicate that up to 100μM SCA did not adversely affect cell growth.



In addition when treated with SCA, the secretion of inflammatory mediators was significantly less in the LPS-stimulated cells. IL-10 concentration of treated cells was 69.6% lower than untreated cells (p = 0.015), whereas GM-CSF concentration was 46% lower in treated cells (p=0.0007).



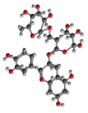
References

1. Ellulu MS, Patimah I, Khaza'ai H, Rahmat A, Abed Y. Obesity and inflammation: the linking mechanism and the complications. Arch Med Sci. 2017 Jun;13(4):851–63
2. Blackhall ML, Berry R, Davies N, Walls JT. Optimized extraction of anthocyanins from Reid Fruits' Prunus avium 'Lapins' cherries. Food Chem. 2018 Feb 27;:280–5
3. Alvarez-Suarez JM, et al. Anti-inflammatory effect of Capuli cherry against LPS-induced cytotoxic damage in RAW 264.7 macrophages. Food Chem Toxicol. 2017 Apr;102:46–52.

Acknowledgements
This work could was supported by Reid Fruits and Essential Oils of Tasmania.

Conclusions

Observations from the *in vitro* studies suggest that the sweet cherry anthocyanins are inhibiting production of GM-CSF from the macrophages at a cellular level, which has implications for understanding the mechanism of attenuating inflammation.



Contact Us
Faculty of Health
For further information contact
Phone: +61 3 62267638
Email: Melanie.Blackhall@utas.edu.au
www.utas.edu.au/health